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INNOVATIVE APPROACHES TO STUDY BASOPHIL FUNCTION IN INFLAMMATION

Zenib Aljadi

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Innovative approaches to study basophil function in inflammation

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By

Zenib Aljadi.MD

Principal Supervisor:
Professor Joachim Lundahl
Karolinska Institutet
Department of Medicine, Solna
Unit of Immunology and Allergy

Co-supervisor(s):
Associated professor Aman Russom
Royal Institute of Technology
School of biotechnology
Division of proteomics and nanobiotechnology

Professor Ola Winqvist
Karolinska Institutet
Department of Medicine, Solna
Unit of Immunology and Allergy

Associated professor Anna Nopp
Karolinska Institutet
Department of Medicine, Solna
Unit of Immunology and Allergy

Opponent:
Associated professor Guro Gafvelin
Karolinska Institutet
Department of Clinical Neuroscience
Therapeutic immune design unit

Examination Board:
Professor Berndt Stegmeyer
Umeå University
Department of Public Health and clinical Medicine

Associated professor Sjoerd Haasl
Royal Institute of Technology
Department of Technology and Health
Center for Technology in Medicine and Health

Associated professor Nailin Li
Karolinska Institutet
Department of Medicine
Division of Clinical Pharmacology

To my parents

There are things known and there are things unknown,
and in between are the doors for perception.

Aldous Huxley

ABSTRACT

Basophils are circulating granulocytes. They are very rare and represent less than 1% of peripheral blood leukocytes. Basophils connect the innate and adaptive immune responses by the secretion of a variety of immune-mediators involved in the pathogenesis of many inflammatory diseases mainly allergic reactions and autoimmune diseases. For many years it was difficult to study basophil function due to their rareness in peripheral blood which resulted in scant yields and purity when isolated. This thesis focuses on studies of the basophil function in two inflammation-driven diseases: chronic kidney disease (CKD) and allergy. The investigations have been done using newly developed microfluidic-based lab-on-chip technology and conventional immunological methods. In **paper I**, we investigated the impact of blood-membrane interaction on circulating basophils and neutrophils in hemodialysis patients (stage 5D), using high-flux and low-flux dialyzers. Passage through the low-flux dialyzer, as opposed to high-flux, induced a significant upregulation of CD63 on formyl-methyinoyl-leucyl-phenylalanine (fMLP) and anti-FcεRI antibody stimulated basophils. Furthermore, (fMLP) stimulated basophils significantly upregulated CD63, in patients compared to healthy controls. There were no significant differences in the expression of neutrophil activation markers (CD11b, the active epitope of CD11b, and CD88), when comparing the two dialyzers, or when compared to healthy controls. In **paper II**, we analyzed the expression of activator markers on basophils related to two crucial functions (transmigration and degranulation) in CKD (stage 5D). The CD300a expression was significantly higher in patients following activation by fMLP and anti-FcεRI-ab and the expression of the active epitope of CD11b was significantly higher in patients after lipopolysaccharide (LPS) activation. The CD62L expression was significantly downregulated in anti-FcεRI activated basophils from healthy controls. In **paper III**, we developed a novel microfluidic immuno-affinity based basophil activation test (miBAT) assay. The microfluidic device is capable of isolating basophils directly from whole blood and we analyzed the regulation of CD203c and CD63 in anti-FcεRI activated basophils in healthy and allergic individuals. The microfluidic chip was able to capture basophils from whole blood with an efficiency of 65% and the CD63 expression detected via fluorescent microscope was significantly higher in activated basophils compared to non-activated basophils (negative control), as well as in allergic patients compared to healthy controls in microfluidic chip. The result was comparable to flow cytometry data. In **paper IV** we validated that the miBAT platform can be used for allergy diagnosis. CD63 expression on basophils activated with allergens was detected in microfluidic chip and flow cytometry. The activation was significantly higher compared to non-activated basophils in allergic patients. Basophils from non-allergic individuals did not respond to allergen activation. The microfluidic chip analysis was comparable with flow cytometry data.

In conclusion, this thesis presents new insights on the role of basophils in the inflammatory responses, mainly related to innate immune responses in CKD patients. Moreover, we introduced a novel microfluidics based method (miBAT) to quantify basophil activation in allergic patients. The method has great potential to be used as a point of care for allergy diagnosis.

LIST OF SCIENTIFIC PAPERS

I. **Zenib Aljadi***, Ladan Mansouri*, Anna Nopp, Josefin M. Paulsson, Ola Winqvist, Aman Russom, Mårten Ståhl, Britta Hylander, Stefan H.Jacobson, and Joachim Lundahl.
Activation of Basophils Is a New and Sensitive Marker of Biocompatibility in Hemodialysis.
Artificial Organs 2014, 38(11):945–953.

II. **Zenib Aljadi**, Anna Nopp, Ola Winqvist, Aman Russom, Britta Hylander, Stefan H. Jacobson and Joachim Lundahl.
Altered basophil function in patients with chronic kidney disease on hemodialysis.
Clinical Nephrology, Vol. 88 – No. 2/2017 (86-96).

III. **Z. Aljadi**, F. Kalm, H. Ramachandraiah, A. Nopp, J. Lundahl, and A.Russom.
Microfluidic immunoaffinity basophil activation test for point of care allergy diagnosis
Submitted manuscript

IV. **Zenib Aljadi***, Frida Kalm*, Caroline Nilsson, Ola winqvist, Aman Russom, Anna Nopp# and Joachim Lundahl#.
Microfluidic immunoaffinity basophil activation test: A new tool for clinical diagnosis of allergy.
Manuscript.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Harisha Ramachandraiah, **Zenib Aljadi**, Torbjörn Pettersson and Aman Russom.
Layer-by-layer system based on cellulose nanofibrils for capture and release of cells in microfluidic device.
Submitted manuscript.

Zenib Aljadi, Tharagan Kumer, Sharath Narayana, Joachim Lundahl, and Aman Russom.
Beads based isolation and enzymatic analysis of basophil activation for point of care allergy diagnostics.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AIT	Allergy immunotherapy
AND	Anaphylactic degranulation
APCs	Antigen presenting cells
ATPs	Atopy patch tests
BAFF	B-cell activating factor
BAT	Basophil activation test
BM	Bone marrow
C/EBP	CCATT enhancer-binding protein
CCL2	Chemokine C ligand 2
CKD	Chronic kidney disease
CRs	Complement receptors
CTCs	Circulating tumor cells
CVD	Cardiovascular disease
DBPCFC	Double-blind placebo-controlled food challenge
DCs	Dendritic cells
DEP	Dielectrophoresis
Der p 1	Dermatophagoides pteronyssinus 1
ENPP-3	Ectonucleotide pyrophosphatase/phosphodiesterase
ESRD	End stage renal disease
FcγRIIb	Fc gamma receptor IIb
FcεRI	Fc epsilon receptor I
FMLP	N-formylmethionyl-leucyl-phenylalanine
FPR	Formyl peptide receptor
FPRL1	Formyl peptide receptor-like 1
GATA2	GATA binding protein 2

GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HD	Hemodialysis
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-	Interleukin
ITIM	Immuno Tyrosine Inhibitory Motif
LAMP	Lysosome-associated membrane glycoprotein
LNs	Lymph nods
LTD4	Leukotriene D4
LTE4	Leukotriene E4
MACS	Magnetic activated cell sorting
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MiBAT	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinases
NKCs	Natural killer cells
PAF	Platelet activating factor
PD	Peritoneal dialysis
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PGD2	Prostaglandin 2
PMD	Piecemeal degranulation
PRR	Pattern recognition receptor
RANTES	Regulated on activation normal T cell expressed and secreted

Re	Reynold’s number
ROS	Radix Oxygen Species
SLE	Systemic lupus erythematosus
SPTs	Skin prick tests
Th2	T-helper 2
TLRs	Toll like receptors
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 IMMUNE SYSTEM

The immune system consists of a collection of cells and soluble mediators to protect the host against microbial infections through two types of defense mechanisms, the innate and adaptive immunity. Innate immune response is mediated by pattern recognition receptors (PRR), and mediates blockage or elimination of infections in a rapid immune response. The innate immune system comprises of physical epithelial barriers, leukocytes such as neutrophils, monocytes, macrophages, dendritic cells (DCs), natural killer cells (NKCs), mast cells, eosinophils and basophils, as well as circulating plasma proteins such as complements. Adaptive immune responses are more specialized and mediated by antigen receptors that allow the generation of diverse antigen-presenting repertoire. T-lymphocytes and B-lymphocytes are players in adaptive immunity. The immune system's ability to eliminate foreign microorganisms also suggests it has the potential to harm the body by reacting against harmless antigens or tissues from self. Such responses can cause allergy and autoimmune diseases.

1.2 THE BASOPHIL CELL

1.2.1 Basophil morphology and phenotype

The basophilic granulocyte was discovered at the end of the nineteenth century by Paul Ehrlich, as blood-circulating cells that are 8-10 μm in diameter with a segmented and condensed nucleus, cytoplasm containing basophilic granules (Stone, Prussin and Metcalfe 2010). They are very rare and represent less than 1% of peripheral blood leukocytes. Basophils are able to promote chronic allergy inflammation, to regulate T-helper2 (Th2) cell function and immune cell memory (Zhong et al. 2014, Voehringer 2013, Kawakami 2008) and even to perform as antigen-presenting cells (Kawakami 2008). Basophils express a variety of effector receptors e.g. cytokine, chemokine, complement receptors, prostaglandin receptors (CRTH2), cysteinyl leukotriene (LTD4, LTE4) receptors (Schroeder, Chichester and Bieneman 2009), immunoglobulin Fc receptors (FcεRI and FcγRIIb), and toll-like receptors (TLRs)(Sullivan and Locksley 2009). Many of them are beneficial in dissecting basophil function in inflammation.

1.2.2 Basophil development

Basophils arise from a common granulocyte-monocyte precursor (CD34+) in the bone marrow (BM). The granulocyte-monocyte precursors differentiate into mast cell precursors and basophil precursors. Mast cell precursors develop into mature mast cells and basophil precursors into basophils. The commitment of these precursors to the basophil lineage is dependent on the expression of the transcription factors GATA-2 and the CCATT enhancer-binding protein C/EBP. They control the switching of the granulocyte-monocyte precursor to be differentiated into the basophil precursor or mast cell precursor, conferring to the upregulation and down regulation of GATA-2 and C/EBP. In basophil differentiation, C/EBP is upregulated to induce differentiation and maturation of basophils (Arinobu et al. 2005).

1.2.3 Basophil growth factors

Basophils circulate in the blood stream and have a mature phenotype. The lifespan of basophils is fairly short (60–62h) (Ohnmacht and Voehringer 2009). Interleukine-3 (IL-3) is the main cytokine which promotes differentiation and subsequent survival of basophils in the BM. There are other growth factors regulating basophil formation and development in bone marrow such as interleukin-5 (IL-5) and the granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hirai, Morita and Miyamoto 1992).

1.3 BASOPHIL ACTIVATION

Basophil activation is mediated by an array of signals including, antibodies (IgE, IgG), cytokines (IL-3, IL-18 and IL-33), proteases, TLRs ligands and complement factors (IgE-independent pathway). When activated, basophils degranulate to release a variety of immune-mediators such as histamine, proteoglycans (e.g. chondroitin and chondroitin sulphate), and proteolytic enzymes, which are pre-stored within cytoplasmic granules (Schroeder et al. 2009). They also secrete lipid mediators such as leukotrienes and prostaglandins (LTC4, PGD2). Upon activation basophils produce several important cytokines (IL-3, IL-4, IL-6, IL-13, IL-25), chemokines (RANTES, MIP-1α, MIP-1β, MCP-1) and GM-CSF (Schroeder et al. 2009, Yamaguchi et al. 2009), chemotactic factors known to recruit multiple immune cells e.g. neutrophils toward the inflamed area (Figure 1).

1.3.1 Antibody mediated basophil activation

Basophils express the high-affinity receptors for Immunoglobulin-E IgE (FcεRI) and cross-linking of FcεRI via IgE leads to rapid release of histamines and LTC4 from basophil granules, and production of cytokines. The rapid production of immune modulators links them as significant players during systemic anaphylaxis, which may occur when the allergen cross-links FcεRI-IgE on the basophil surface. Activation of basophils through FcεRI is known as the typical basophil activation pathway (IgE-dependent pathway) (Stone et al. 2010, Steiner, Harrer and Himly 2016) but basophils can also be activated by Immunoglobulin-G IgG. They express IgG receptor (FcγRIIA) (activating receptor) and upon capture of IgG-allergen complexes, basophils release a platelet-activating factor (PAF), which increases vascular permeability and occasionally leads to anaphylaxis (Tsujimura et al. 2008). In addition, basophils express another IgG receptor (FcγRIIB) (inhibitory receptor), which is coupled to the anti-inflammatory response of basophils.

1.3.2 Toll Like Receptor (TLR) mediated basophil activation

Basophils constitutively express mRNA of several Toll-like receptors (TLRs), including TLR2, TLR4, TLR9 and TLR10. TLR mRNA expression in basophils is generally less prominent than that in neutrophils and monocytes, but basophils express significantly higher levels of TLR2 and TLR4 mRNA (Komiya et al. 2006). TLR4 on basophils are involved in the pathogenesis of infection-induced exacerbation of allergic inflammation by modulating basophil functions.

1.3.3 Cytokine and chemokine mediated basophil activation

Activated basophils secrete cytokines e.g. IL-4 which initiate the differentiation Th2 lymphocyte and of IgE-secreting B-lymphocytes (Suurmond et al. 2014b). Accumulated evidence indicates that IL-3 is the most potent activator of basophils. Pretreatment of basophils with IL-3 results in marked enhancement of histamine release initiated by anti-IgE and formylmethionyl-leucyl- phenylalanine (fMLP) activation of basophils (Karasuyama et al. 2009). IL-3 signaling activation enhances basophil function by secretion of IL-4 and IL-13 after IgE-dependent stimulation (Schroeder et al. 2009, Borriello et al. 2015a). IL-3 regulates the expression of activator markers in basophils such as the (CD203c) piecemeal degranulation marker (Sturm 2011, Gentinetta et al. 2011). Basophils express IL-1 family receptors, IL-8 and IL-33 cytokine receptors and respond to IL-18 and IL-33; they are capable of activating basophil and augment Th2 responses (Kroeger, Sullivan and Locksley 2009). IL-33 is associated with IL-4, IL-13 and IgE production of basophils (Humphreys et al. 2008). Chemokines are also able to directly activate basophils. The most potent secretagogue chemokine inducing histamine release from basophils is MCP-1/CCL2 (Schleimer et al. 1989).

1.3.4 Protease mediated basophil activation

Basophil activation is known to be mainly antibody- and cytokine-dependent, allergens and parasite-derived antigens are also capable of activating basophils directly. The antigens with protease activity activate basophils in an IgE-independent manner. Some allergens with an active protease antigen such as house dust mites (Dermatophagoides pteronyssinus 1) (Der p 1) activate basophils and induce the production of IL-4 and IL-13 from basophils in the absence of antigen-specific IgE interaction (Phillips et al. 2003). In addition, papain, a cysteine protease allergen, can activate basophils and induce IL-4 and IL-6 production from murine basophils, as well as inducing the migration of basophils to lymph nodes (LNs) in vivo (Sokol et al. 2008). It has also been shown that helminth-derived proteases activate basophils with the same manner of allergen proteases. The underlying mechanism of protease basophil activation is not clearly known but it has been suggested that basophils express an unknown receptor recognized by proteases antigens (Chen et al. 2009).

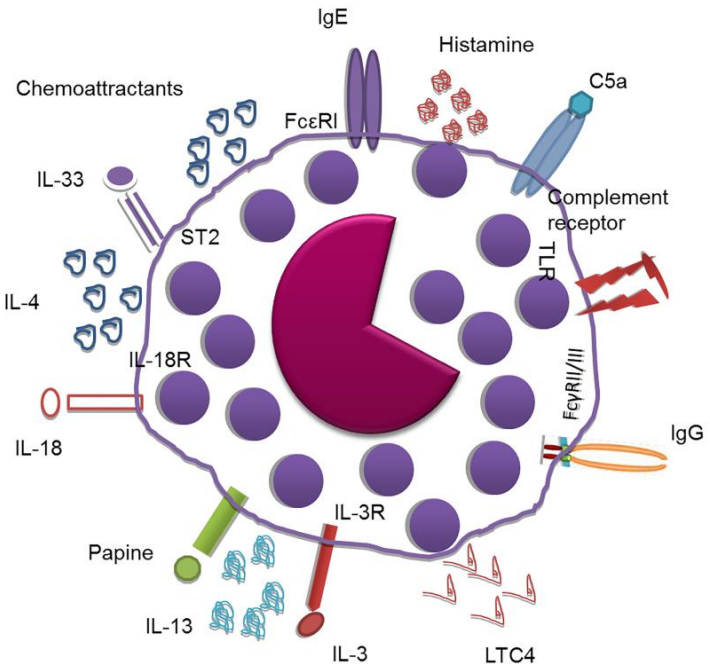


Figure1. *Basophil activation responses. Basophils express a variety of effector receptors and upon activation basophils secret effector mediators involved in the inflammatory responses.*

1.4 BASOPHIL DEGRANULATION

Degranulation is a cellular process that releases immune mediators from secretory granules found in the cytoplasm of several cells involved in the immune responses, including granulocytes (neutrophils, basophils, and eosinophils) and mast cells (Kraft et al. 2005). There are two types of basophil degranulation: piecemeal degranulation (PMD) and anaphylactic degranulation (AND). During anaphylactic degranulation, the cells undergo rapid morphologic changes and exocytosis of intracellular granules, while in piecemeal degranulation cells secrete granule contents without exocytosis (Dvorak et al. 1983).

1.4.1 IgE-dependent basophil degranulation

The expression level of human basophil FcεR1 correlates with free IgE levels. FcεRI is a tetrameric (αβγ2) receptor where the extracellular domain of α subunit is the binding site of the Fc portion of IgE in basophil surface. Crosslinking of FcεRI by polyvalent antigens (i.e. allergens) or immune complexes recognized by bound IgE leads to aggregation of FcεRI-IgE in basophil surface, activation of downstream intracellular signaling cascade regulates calcium (ca+) mobilization in basophils, granule exocytosis, histamine release and cytokine production (Karasuyama et al. 2010).

1.4.2 IgE-independent basophil degranulation

Degranulation of basophils and histamine release can be induced by IgE-independent manner activation such as anaphylatoxins C3a and C5a, and the chemotactic tripeptide formyl-methionyl-leucyl-phenylalanine (fMLP)(Chirumbolo et al. 2008).

Formyl-methionyl-leucyl-phenylalanine (fMLP) is N-formyl peptides and they are the biologically relevant ligands for formyl peptide receptors (FPR) which are G protein-coupled

receptors. Basophils express the high affinity receptor FPR and its homologue formyl peptide receptor-like 1 (FPRL1), fMLP binds on basophils, that activates subsequent intracellular signaling pathways such as the MEK-ERK pathway and leads to chemotaxis and release of mediators such as leukotriene C4 (LTC 4) and histamine (Miura and MacGlashan 2000, de Paulis et al. 2004).

Human basophils express complement-binding receptors e.g. CR3, CR4 and CD88 which is a receptor of C5a complement factor, and induce histamine release from basophils in an IgE-independent manner (Schulman et al. 1988).

1.4.3 Basophil expression of degranulation markers

Basophil activation leads to upregulation of activator markers in basophil cell surface (CD203c and CD63) correlated to degranulation. As well as upregulation of the inhibitory marker which inhibits basophil degranulation (CD300a) (Figure 2).

1.4.3.1 CD203c expression on basophils

CD203c is an ectonucleotide pyrophosphatase/phosphodiesterase (ENPP)-3, CD203c has been known to be specific for basophils, mast cells and their progenitors (Buhring et al. 1999). CD203c is expressed constitutively on resting basophils at low to intermediate levels and its expression is rapidly upregulated following activation of basophils. CD203c has been considered to be a piecemeal degranulation marker and its expression has been used as a marker to assess the basophil activity (MacGlashan 2010, Crivellato et al. 2003).

1.4.3.2 CD63 expression on basophils

CD63 is a membrane protein of the Lysosome-associated membrane glycoproteins (LAMP) family, a family of tetraspanin proteins often found to be involved in vesicle fusion. CD63 is located in the same granules as histamine and is often used as a marker of basophil activation and degranulation (Thyagarajan et al. 2012).

1.4.3.3 CD300a expression on basophils

CD300a is regarded as an inhibitory marker for degranulation. The inhibitory effect of CD300a in basophil degranulation is thought to arise from an Immuno Tyrosine Inhibitory Motif (ITIM) positioned within the intracellular domain of the receptor forming a reducing site for the inositol 5-phosphatase (SHIP). SHIP-1 is involved in reducing basophil responses following FcεRI activation However, CD300a is expressed with higher levels on IgE dependent basophil activation, while it is expressed with low levels in independent IgE activation (Gibbs et al. 2006, Gibbs et al. 2012).

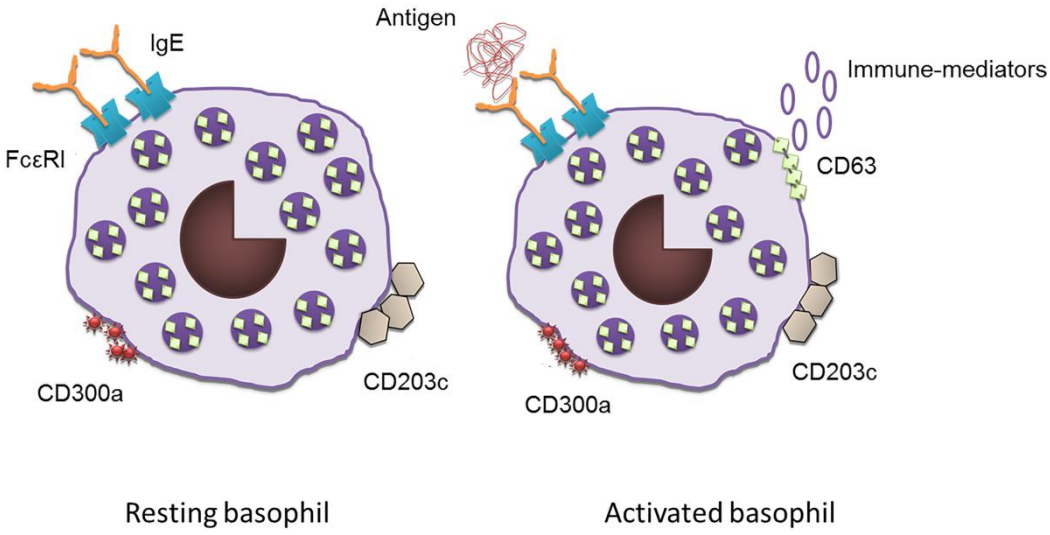


Figure 2. *Degranulation markers expression on basophils. Upregulation of CD203c, CD63 and CD300a in activated basophils.*

1.5 BASOPHIL TRAFFICKING

Basophils circulate predominantly in the blood, but they can also be found trafficking to lymph node (LN) and spleen. They are also recruited to sites of inflammation and involved in the inflammatory responses (Schmitz et al. 2005). Basophils express adhesion molecules and chemokine receptors which regulate cell transmigration (Figure 3).

1.5.1 Basophil adhesion

Basophil adhesion to vascular endothelial cells is regulated and enhanced by several cytokines such as IL-3, IL-5 and GM-CSF (Bochner et al. 1990, Yoshimura-Uchiyama et al. 2003). Several receptors and mediators involved in basophil migration have been characterized. A family of adhesion receptors termed "selectins" (CD62L) is thought to mediate the initial attachment and rolling step; a number of chemotactic mediators have been implicated in the activation step, and integrins expressed on basophils binding to adhesion receptors (CD11b/CD18) belonging to the immunoglobulin superfamily are implicated in the firmer adhesion step. Enhancement of basophil adhesion by several cytokines is mediated by upregulation of surface β2 integrin (CD11b/CD18) expression.

1.5.1.1 CD11b expression on basophils

CD11b is an integrin family member which pairs with CD18 to form the CR3 heterodimer. CD11b is expressed on the surface of many leukocytes including monocytes, neutrophils, natural killer cells, macrophages and basophils. Functionally, CD11b regulates leukocyte adhesion and migration to mediate the inflammatory response. CD11b antibody studies have shown the protein to be directly involved in cellular adhesion, although migration can only take place in the presence of the CD18 subunit. It has been shown that the conformational changes in CD11b after activation may be more relevant to CD11b functional capacity than total cell surface expression. Conformational activation of CD11b exposes a neo-epitope

within the I domain known as CBRM1/5, and facilitates the actual binding capacity (Olsson et al. 2009, Kanayama et al. 2004).

1.5.1.2 CD62L expression on basophils

CD62L is an adhesion molecule involved in early leukocyte attachment to the endothelium at sites of inflammation and is rapidly shed after neutrophil and monocyte activation (Monteseirin et al. 2005, Xu et al. 2008). In a mice model study it was shown that basophils in systemic lupus erythematosus (SLE) upregulate CD62L (Charles et al. 2010). The cytoplasmic tail of CD62L has been reported to regulate shedding, microvillus positioning and the tethering/rolling mediated by interaction with at least three different proteins, including calmodulin, α-actinin (a member of the ezrin/radixin/moesin (ERM) family of membrane-cytoskeleton cross-linkers), and protein kinase C isoenzymes. Disruption of these interactions may reduce the shedding or inhibit tethering/rolling efficiencies (Jung and Dailey 1990, Dwir, Kansas and Alon 2001).

1.5.2 Basophil transmigration

Basophils expressed transcripts of various chemokine receptors such as CCR1, CCR2, CCR3, CCR5, CXCR1 and CXCR2 (Bischoff et al. 1992, Yamada et al. 1997). The most potent basophil migration is induced by eotaxin/CCL11. In addition, migration of basophils toward eotaxin was enhanced by very weak FcεRI-crosslinking activation. Basophil transmigration is regulated by several cytokines e.g. IL-3, GM-CSF, IL-5 and IL-4. Eotaxin binds specifically and exclusively to CCR3, whereas RANTES binds to CCR1 and CCR3, although with higher affinity for CCR1 both chemokines induced strong basophil Trans Endothelial Migration (TEM) (Iikura et al. 2004). Moreover, the chemokines IL-8 and RANTES induced basophil migration. Other chemokines, including MCP-1 displayed weak basophil attracting potency. In addition, studies suggest that matrix metalloproteinases (MMP-9) is expressed on the basophils, and enhances the expression of β2 integrin on basophils (Suzukawa et al. 2006).

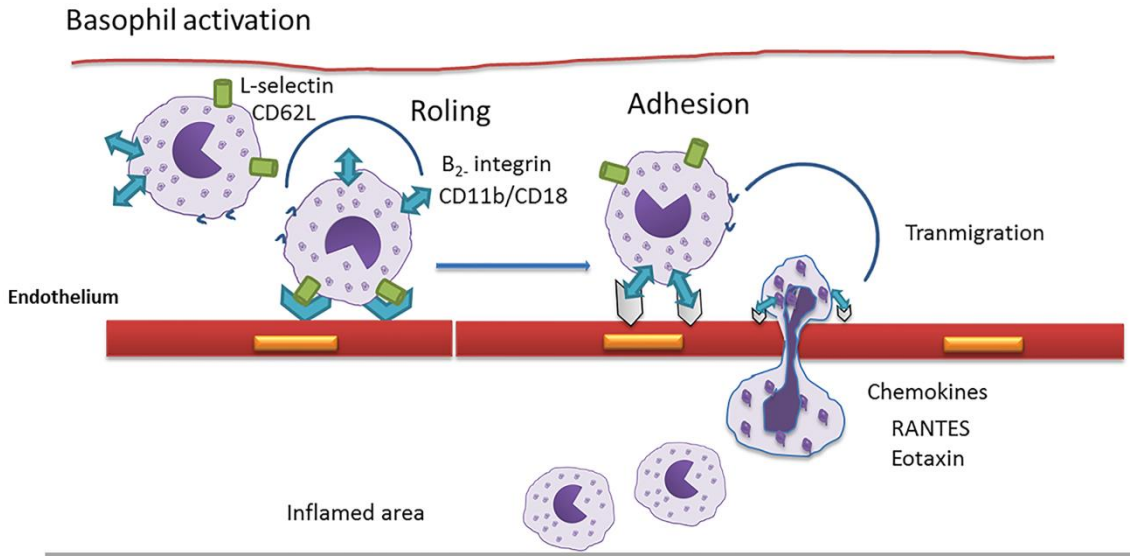


Figure 3. *Basophil transferring process from blood circulation to inflammation area. Expression of adhesion molecules on basophils.*

1.6 CHRONIC KIDNEY DISEASE AND BASOPHILS

1.6.1 Definition and classification of chronic kidney disease (CKD)

Chronic kidney disease is a worldwide public health problem, associated with progressive disturbance of the structure and function of the kidney tissue. Chronic kidney disease is linked to adverse outcomes of kidney failure, cardiovascular disease (CVD), and premature death (Levey et al. 2005). CKD is defined as kidney damage or glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for 3 months or more, irrespective of cause. Kidney damage in many kidney diseases can be determined by the presence of albuminuria and albumin/creatinine ratio >30g/mol (Moe et al. 2006).

CKD classification based on GFR level (Table 1):

Stage 1	Kidney damage with normal or increased GFR ≥90
Stage 2	Kidney damage with mildly decreased GFR 60 to 89
Stage 3	Moderately decreased GFR 30 to 59
Stage 4	Severely decreased GFR 15 to 29
Stage 5	Kidney failure <15 or dialysis

In patients with end stage renal disease (ESRD) treated with renal replacement therapy which includes dialysis, either hemodialysis (HD) or peritoneal dialysis (PD), and kidney transplantation.

1.6.2 Dialysis therapy

Dialysis is a procedure used to filter the blood wastes, fluids and salts that accumulate in the body due to kidney function failure. In peritoneal dialysis (PD) a peritoneal membrane lines the abdominal wall and works as a filter, and requires the placement of a catheter in the peritoneal cavity to allow fluid to be instilled and drained out. The filtration of wastes and excess water by PD procedure depends on different principles such as diffusion, osmosis, and ultrafiltration (Fenton et al. 1997). In hemodialysis (HD) the blood is circulated through an artificial kidney with two compartments: blood and dialysate, separated by a thin semi-permeable membrane. Waste such as urea and excess water passes from the blood side to the dialysate side through a dialyzer membrane and is discarded into the drain. The cleaned blood is reverted to the patient, through insertion of access to the circulatory system such as arterio–venous fistula, arterio–venous graft, temporary catheter and long–term catheter (Ronco 2015). However, as a consequence of bio-incompatibility, blood-dialysis membrane contact may lead to a variety of adverse reactions in immune cells function and plasma proteins.

1.6.2.1 Hemodialysis and biocompatibility

The biocompatibility is influenced by the interaction between the blood and the treatment materials. The surface material of the dialyzers and the membrane permeability properties are the main causes of alterations in leukocytes function, cytokine production, phagocytic efficiency, apoptosis, Radix Oxygen Species (ROS) production, and complement activation (Banche et al. 2006). The modified and synthetic materials were introduced to improve the biocompatible properties of the dialyzers. The permeability of the membrane contributes to the over-all biocompatibility properties. High flux dialyzers (with large pore size and higher ultrafiltration coefficients) cleared large molecules, such as β2-microglobulin and toxins with enzymatic and metabolic inhibitory effect, compared to low flux dialyzers (with smaller pore size and lower ultrafiltration coefficients)(Chauveau et al. 2005), but the clinical consequences in terms of morbidity and mortality of long term use are still debated.

1.6.2.2 Basophils and biocompatibility

Hemodialysis patients may develop anaphylactic reactions. The underlying mechanism involved in hemodialysis-associated anaphylactic reaction can be related to IgE-mediated reaction due to cross linking of FcεRI on the surface of basophils, and rapid release of histamine caused by sensitization by materials used during dialysis. Also non-IgE-mediated anaphylactic reaction may occur by accumulated C3a and C5a. Furthermore, the reaction might also be primed by accumulation of the potent vasodilator bradykinin, which induces histamine release from basophils (Ebo et al. 2006). During hemodialysis, IL-1β and TNFα cytokines are secreted from activated monocytes which induce histamine release from basophils (Subramanian and Bray 1987).

1.6.3 Basophil function in CKD

Involvement of basophils in autoimmune kidney diseases, such as lupus nephritis (LN), has previously been proposed. Formation of immune complexes may activate basophils to secrete IL-4, initiate Th2 differentiation, B cell proliferation, plasma cell differentiation and immunoglobulin production, and thereby be responsible for the ongoing humoral immune response in affected tissues. The auto-reactive immune complexes activate basophils and upregulate the cell surface expression of markers, such as CD203c and CD63 (Bosch et al. 2011, Mack and Rosenkranz 2009). Circulating basophils have increased CD62L expression, which permits leukocyte homing to peripheral lymphoid tissues. Basophils also express the membrane-associated B-cell activating factor (BAFF), showing a potential role for lymph node basophils in B-cell survival and differentiation. In addition, basophils from mice lymph nodes and spleen have high expression of MHC II. Higher expression of MHC II and/or BAFF in the lymph nodes and spleen could permit communication with T and B cells (Charles et al. 2010). Activation of TLR pathways has been implicated in various renal diseases, including acute kidney injury, ischemia–reperfusion injury, allograft rejection and immune complex nephritis (Czyzyk 2006). The circulating immune complexes in autoimmune kidney disease bind to specific receptors on cell surfaces such as Fcγ receptors (FcγR) and TLRs activate cells (Gollapudi et al. 2010). TLR9 has been found to be expressed in the intracellular compartments of basophils and to be involved in the

activation of peripheral blood basophils in patients with systemic lupus erythematosus (Pellefigues and Charles 2013).

1.7 ALLERGY AND BASOPHILS

1.7.1 Allergic diseases

Allergy is a worldwide medical problem; the prevalence of allergic diseases in westernized countries is 25-30% of the population (Pawankar et al. 2012). Allergy is a multifactorial disease resulting from the combination of genetic susceptibility and environmental factors. Allergic reactions vary in severity from mild itching to life threatening anaphylaxis. The broad spectrum and the potentially serious nature of symptoms negatively affect the quality of life for the affected individuals. Allergic diseases include food allergies, certain forms of asthma, rhinitis, conjunctivitis, angioedema, urticaria, and eczema, eosinophilic disorders, including eosinophilic esophagitis, drug and insect allergies (Pulendran and Artis 2012).

1.7.2 IgE-mediated allergic response

The IgE-mediated allergic response can be observed in two phases; sensitization phase and effector phase. The sensitization phase (formation of memory T cell, B cell and production of IgE antibodies of allergens) after uptake and processing the allergen by antigen presenting cells (APCs). The effector phase is divided into an acute response (5-15 minutes) which may be followed by a late reaction hours after allergen exposure (Larche, Akdis and Valenta 2006).

1.7.2.1 Immediate allergic response and basophils

Upon re-exposure to an allergen after the sensitization process, the immediate allergic reaction is initiated by allergen cross linking of IgE-ab bound to FcεRI on the basophil surface. Basophil activation is followed by degranulation and release of immune-modulators such as histamine. Histamine causes symptoms depending on the site where sensitization and subsequent exposure have occurred. Together with newly produced cytokines (IL-4. IL-13) and chemokines, these mediators promote leukocyte recruitment to the site of inflammation and contribute to the late allergic response (He et al. 2013, Mukai et al. 2005).

1.7.2.2 Delayed allergic response and basophils

Delayed allergic reaction typically develops after 2–6 h of allergen exposure. It is usually preceded by acute allergic reactions and secretion of inflammatory mediators, which promote migration of cells e.g.TH2 cells, eosinophils, basophils and other leukocytes. This IgE-mediated reaction leads to aggregation of FcεRI, increased allergen uptake and recognition of allergen-derived peptides by specific memory CD4+T-cells, causing their reactivation and clonal expansion, which leads to aggravation of inflammatory responses. Delayed allergic reaction can occurs in the lungs in severe asthma, in the upper respiratory tract in individuals with allergic rhinitis and in the skin of individuals with atopic eczema. Promoted type 2 immune response enhances production of cytokines and leads to powerful late inflammatory response (Cromheecke, Nguyen and Huston 2014, Katz 1978) (Figure 4).

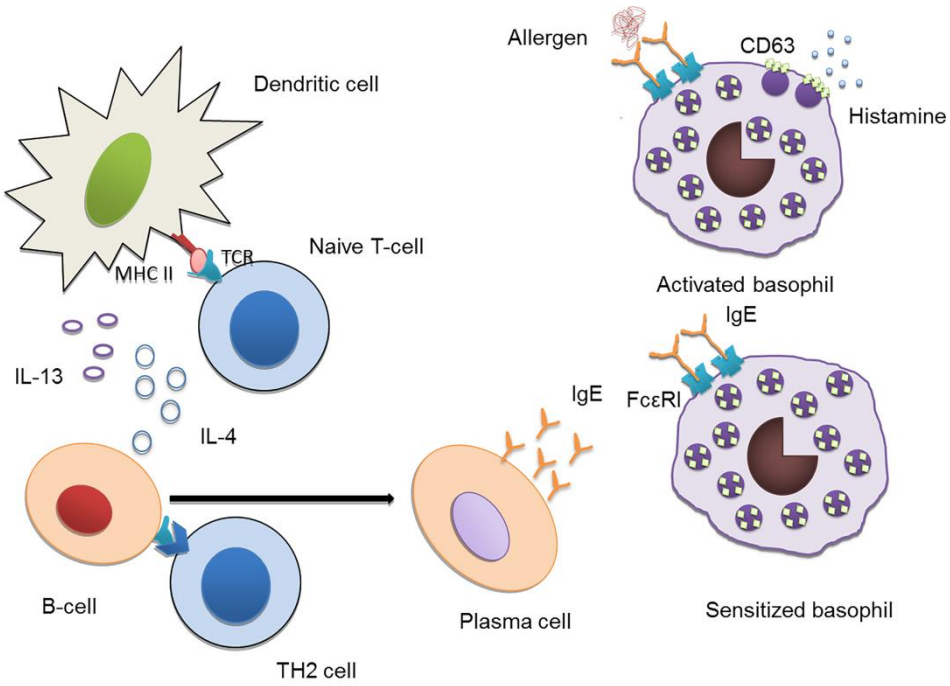


Figure 4. *Schematic illustration of the role of basophils in IgE-mediated allergic reaction.*

1.7.3 Allergy diagnosis

Clinical history and examination are the first-line of allergy diagnosis. The evaluation of a patient with suspected allergy starts with obtaining a systematic clinical history that reflects the indicative symptoms of allergic reactions. The clinical manifestations of allergy vary and correlate to entry site of allergen into the body and the onset of symptoms indicating the type of allergic reaction. However, the physical examination may indicate whether the reaction is acute or a chronic response. The clinical history and examination lack sufficient specificity and sensitivity to establish the diagnosis. Therefore, in vivo (skin testing) and in vitro (allergy-specific serum IgE-ab) investigations of sensitization are essential assistant tools for allergy diagnosis.

1.7.3.1 In vivo testing

Skin prick tests (SPTs) are a fast method of judging sensitization using mast-cell reactivity as a read-out for IgE-ab to suspected allergens. Commercially prepared allergen extracts can be used. SPTs are less expensive than in vitro testing. Skin testing can be safely performed in patients of any age; it causes the patient mild discomfort, and yields results within 15 minutes (Foong et al. 2016). Intradermal testing (ID) is another in vivo form of testing for allergens but is not recommended in the diagnosis, due to the high rate of false positive results, and the high risk of systemic life-threatening reactions (Bernstein et al. 2008). Atopy patch tests (ATPs) involve the topical application of a solution containing allergens to the skin for 48 hours. However, ATP is not routinely recommended for example in patients with suspected food allergy (Nowak-Wegrzyn et al. 2008). Moreover, in vivo testing is contraindicated or ineffective in some cases (extended dermatitis, dermographism, severe atopic dermatitis, medication that inhibits cutaneous reactivity).

1.7.3.2 In vivo challenge tests

The in vivo challenge test is mainly used for food allergy. Supervised food challenge controlled protocols where the suspected food allergens are introduced into the patient under a clinical observation. They are sometimes required for the absolute diagnosis of food allergy, in which a double-blind placebo-controlled food challenge (DBPCFC) is the most accurate form of challenge. Food is selected for testing based upon the history and the results of skin and/or in vitro testing (Sampson et al. 2012). Challenge tests can also be performed for airway allergic reaction (e.g. asthma) and nasal allergic rhinitis diagnosis (Charron and Pakhale 2016, Steveling et al. 2015). The challenge tests are time consuming, resource-intensive and run the risk of inducing systemic severe allergic reaction.

1.7.3.3 In vitro testing

Analysis of IgE-ab in serum is an important adjunct tool in the accurate identification of allergens. There are different detection systems for IgE-ab, e.g. HYTEC-288 (HycorBiomedical), Immulite (Siemens) and ImmunoCAP (Phadia) (Boyce et al. 2011). The in vitro tests should be correlated with clinical history; higher amounts of IgE-ab are more likely to indicate clinical reactivity. However, the value of IgE-ab levels contrasts within wide ranges e.g. in food allergy age, time since last ingestion of suspected food, and other associated disorders affect the IgE-ab level (Sampson 2001). Both in vivo and in vitro testing detect sensitization, not clinical allergy; they cannot predict prognosis or severity of subsequent reactions.

1.8 BASOPHIL FUNCTIONAL ANALYSIS

1.8.1 Basophil analysis techniques

Basophils play an important role at the crossroads of innate and acquired immunity. Despite their importance, research on basophils was hindered for a long time due to the rareness of basophils in peripheral blood which often resulted in scant yields and difficulty in their isolation. Moreover, only one genetically homogeneous human basophilic-like cell line, KU812 (Kishi 1985) is available. Furthermore, it has functional limitations including a variable cell surface expression of FcεRI and low grade of granulation (Jensen et al. 2005). This fact has led to the use of time-consuming and expensive methods for the purification of primary human basophils from whole blood or buffy coats having to be used, which has greatly limited in vitro studies of basophil biology. Given the recent advances in determining the biological role of basophils in mice using in vivo models, a technique that enables the generation of functional mouse basophils with a normal phenotype is vital. Several attempts have been made to develop basophil isolation and functional analysis but in most cases they have proved to be expensive and time-consuming approaches, such as bead-based isolation, immuno-selection of basophils by monoclonal antibodies and sorting by flow cytometry.

1.8.2 Bead-based basophil functional analysis

Bead-based cell isolation and analysis has been used for different cell fractions. Contaminated cells are able to secret the same cytokines or express the same surface

receptors as basophils and this interferes with the determination of these factors for basophils. Therefore, it is essential to obtain highly purified basophils for investigations where interference by other cells must be avoided. Several methods have been developed over time for the purification of the human basophil from blood and buffy coat in terms of purity, integral cell function and time of cell isolation, using both positive and negative isolation procedures (Gibbs et al. 1997, Gibbs, Papenfuss and Falcone 2008, Bjerke et al. 1993). Cells isolated by beads undergo a gradient separation followed by a purification step with selective antibodies on magnetic beads. The isolated basophils have been used for the functional analysis and gene expression of basophils in many studies (Borriello et al. 2015b, Abdullahi et al. 2016).

1.8.3 Flow cytometry approach of basophil analysis

In vitro studies of basophils obtained from peripheral blood include separation. Physical separation of basophil cells from whole blood or buffy coats can be done by immuno-selection using monoclonal antibodies. Followed by basophils isolation by either a cell sorter (Yang et al. 2010) or electronically captured by a flow cytometer (Chirumbolo et al. 2008). Flow cytometry can identify these cells and follow their response upon stimulation or inhibition; cells are captured as electronic events and plotted as a purified population in the so-called dot plot diagram.

1.8.3.1 Flow cytometric basophil functional characterization

Several phenotypic markers can be used to isolate basophils from other leukocytes. Basophils are typical CD45 cells in the lymphocyte area, express IL-3 receptor α-chain (CD123), and do not express HLA-DR and are used as a strategy for flow cytometric basophil gating. Many other strategies have been used to identify basophil biology e.g. (IgE+, CD3-/CRTH2+; CD3-/CCR3+; CD14-/CD13+, CD45+/CD203c+) (Chirumbolo, Ortolani and Vella 2011). When basophils have been gated, they can be analyzed for their response to several stimuli by detection of upregulation or downregulation of different surface markers that correlate to different basophil functions such as CD63, CD203c, CD193 (CCR3), CD164, CD107a, CD62L, CD69 and CD11b (Florian et al. 2006, Hennersdorf et al. 2005, Komiya et al. 2006). Furthermore, flow cytometry can be used for the analysis of intracellular protein regulation after activation, such as signaling proteins or cytokines (Freer 2014, Verweij et al. 2010), by targeting them with fluorochrome conjugated antibodies. The flow cytometry assessment of basophil activation upon allergen challenge is the basic principle of the basophil activation test (BAT), a method capable of detecting antigen-dependent cellular response without any risk to the patient used in the allergy diagnosis (Wedi and Kapp 2010).

1.8.3.2 Basophil activation test (BAT)

The basophil activation test (BAT) is a flow cytometry based cellular assay that measures the activation of basophils upon allergen stimulation.

1.8.3.2.1 Technical characteristics of flow cytometry based BAT

BAT can be used as a diagnostic technique to diagnose and monitor allergic patients. The activation response can be measured at a single cell level by using fluorochrome-bound monoclonal antibodies (mAbs) for specific activation markers. Currently, two activation markers, CD63 and CD203c, are commonly used for diagnostic purposes. Upon basophil activation, these two markers are upregulated, but they have different characteristics. CD63 has been used as a degranulation marker upon anaphylactic stimulation (Abuaf et al. 2008). Although CD63 is also expressed on platelets, eosinophils, and monocytes, CD63 expression on basophils can be identified using additional stains for basophil markers such as IgE, CD123, CCR3, CRTH2, and CD203c (Rouzaire et al. 2012). CD203c can also be used as an identification marker since it is exclusively expressed on basophils, and this expression is related to piecemeal degranulation of basophils (Hino et al. 2014). For the BAT procedure, fresh whole blood is withdrawn. Anti-FcεRI mAb, fMLP and anti-IgE are used as a positive control and stimulation buffer alone as a negative control. If subjects do not respond to a positive control (called non-responders), then their BAT results cannot be interpreted and have to be rejected in the analysis.

1.8.3.2.2 Clinical application of BAT

BAT is used as an ex vivo provocation test for allergy diagnosis. It can be performed at the same time as sIgE, and in general precedes in vivo provocation tests, for example, oral food, drug, or bronchial challenge. It is clinically evaluated for the diagnosis of different allergy triggers e.g. inhalant allergy, food allergy and drug allergy. Furthermore, BAT has been used to monitor allergic patients under allergy immunotherapy (AIT) and anti-IgE treatment by measuring the change of basophil sensitivity related to allergen concentration by performing basophil allergen threshold sensitivity (CD-sens) (Hoffmann et al. 2015). Furthermore, BAT has been used to investigate basophil activation in autoimmune diseases such as lupus nephritis (LN) patients by measuring the change in CD203c and CD63 expression in basophil surface after activation of FcεRI with autoreactive IgE complexes (Pellefigues and Charles 2013). Nevertheless, the cost and technical requirements for the operation and maintenance of flow cytometry and the long time for pre-processing and pre-labeling of the sample before flow cytometric analysis have limited its reach for extended clinical application.

1.9 MICROFLUIDIC BASED APPROACHES

1.9.1 Introduction of microfluidics

Microfluidics is a field of research that deals with behavior and manipulation of fluids, generally on the microliter to pico-liter scale, using channel dimensions constrained to hundreds of micro-meters. Microfluidics enables cellular and molecular studies by miniaturization of traditional macroscale methods to overcome several limitations of conventional bio-separation methods. Microfluidic technology reduces the required amounts of reagents, less waste, rapid analysis, high throughput, and less manufacturing cost (Whitesides 2006). From a biological point of view, microfluidics can partly reproduce the in vivo-like molecular and physical microenvironments of cells. Furthermore, microfluidic

systems allow the integration of many steps into a single chip to reduce the errors related to liquid handling or to obtain multiple data points from a single experiment. In general, in microfluidics, certain characteristics of the fluid flow become prominent, such as laminar flow. The fluid phenomena affect how microfluidic devices can be prepared and used (Dittrich, Tachikawa and Manz 2006, Ben-Ari et al. 2013, Dostovalov et al. 2011).

1.9.2 Fluid dynamics

In microfluidics where fluidic resistance and surface tension is dominant, the fluid flow is typically laminar, where viscous losses dominate over inertia losses, and mixing is based on diffusion.

1.9.2.1 Reynold's number

The Reynold's number (Re) is used to describe the fluid flow conditions. Re is a function of velocity and hydraulic diameter which depends on the channel's cross-sectional geometry. Re <2300, indicates a laminar flow that is governed by Newton's viscosity law (Yakhot 2014). In laminar flow fluid particles move along paths in thin layers in which one layer slides upon one another. As Re approaches 2300, the fluid starts to show signs of turbulence, and as Re becomes greater than 2300 the flow is considered to be turbulent (Figeys and Pinto 2000, Yang and Li 1997).

1.9.2.2 Laminar flow

In microfluidics, Re is typically very small; in the order of 0.1. Laminar flow is a condition in which the velocity of a particle in a fluid stream is not a random function of time. In the micro-channels, the flow is almost always laminar (Pandey, Chaube and Tripathi 2015). In laminar flows, the fluid stream flows in parallel layers and mixing between the layers is based on diffusion. Diffusion between laminar streams has been used for sorting particles by size (Brody and Yager 1997, Hatch et al. 2001).

1.9.2.3 Diffusion

Diffusion is the movement of a concentrated group of particles in a volume over time so that the average concentration of particles in the volume is constant. Since distance varies in relation to the power square, diffusion becomes very important on the microscale. The diffusion time is small in the micro-channels; therefore, it can be used to generate a concentration gradient, which is used for a variety of biological assays (Jeon et al. 2000).

1.9.2.4 Fluidic resistance

The fluid resistance in the micro-channels is also one of the important factors affecting the fluid dynamic in a microfluidic chip. The flow rate within a microchannel is expressed as, where Qv is the flow rate, DP is the drop in pressure across the channel, and R is the channel resistance (Minucci et al. 2014). The fluid resistances in the micro-channels differ according to the geometry of the channels.

1.9.2.5 Surface tension

Surface tension is an important force in the microscale. Surface tension is the result of consistency between liquid molecules at the liquid/gas interface. Micro-channels with dimensions on micro-meters are used; the lengths liquids will travel based on capillary forces alone are significant. Surface energies have been used in microfluidics by creating virtual walls (Zhao, Moore and Beebe 2001) as well as pumping.

1.9.3 Microfluidic chip fabrication technologies

Soft lithography is the most common method used for micro-fabrication of microfluidic devices. Briefly, a design is drawn using computer-aided design (CAD) software and printed at high resolution into a photomask which is a transparent sheet (Xia et al. 1999). The pattern is transferred to a substrate by photolithography. In photolithography, a substrate (the master) is coated with a photosensitive polymer layer and exposed to UV light through the photomask. During development, photoresist crosslinked to the substrate surface remains, while UN- crosslinked or cleaved photoresist areas are washed away. The standard photolithography has a resolution of approximately 1-2 μm, but there are other lithography methods used with higher resolution up to sub-100 nm such as E-beam, ion beam, and dip-pen lithography. When using a negative photoresist, SU-8, the master substrate is ready to be used for molding. Alternatively, the pattern is transferred into the substrate material using a wet or dry etch process. Currently, the most common material used for fabricating microfluidic chips for biological applications is polydimethylsiloxane (PDMS), an elastomer that can be easily molded into microstructures and micro-channels. The mechanical and physical properties of PDMS are highly valuable for fabricating microfluidic chips. PDMS is transparent, biocompatible, and gas-permeable, which make it appropriate for molecular and cellular studies (Piruska et al. 2005, Belanger and Marois 2001).

1.10 MICROFLUIDIC BASED CELL ISOLATION

Microfluidics has been used for the isolation and analysis of a variety of cells. Cells are isolated from a large population of other cell types based on one or several properties. Multiple separation techniques have been used to isolate the cells according to their physical properties such as cell size, deformability, compressibility, shape, density, size, surface properties; electrical polarizability, magnetic susceptibility and refractive index have been considered as biomarkers. In general, cell separation is categorized into two types: active and passive techniques.

1.10.1 Active cell separation techniques

As the name implies, active separation techniques require the addition of active forces to operate. External forces are used to utilize the particles' properties for separation such as dielectric, optical, magnetic and acoustics separation.

Briefly, dielectrophoresis (DEP) is an unlabeled technique (immuno-labeling of cells is not needed), which has been widely studied in microfluidics. DEP has been demonstrated to be capable of separating cells based on the variations in the dielectric properties of different

cell types. However this method is relatively slow, that affects the throughput of cell separation (Yang and Li 1997, Shamloo and Kamali 2017).

Magnetophoresis is implemented by applying an external magnetic field and depends on the magnetic properties of different cells. This method has been used to isolate blood cells and sort macrophages from monocytes due to their dissimilar internalization rate of iron nanoparticles (Zhu et al. 2012). Magnetophoresis is often combined with immuno-labeling of cells with paramagnetic micro-and nanoparticles for continuous separation of cells.

Optics has been used to manipulate cells. Recent advances in microfluidic techniques have led to the development of micro-cytometry. Cell sorting based on size (protein microcapsules) and refractive index (separating polymer from silica spheres) has been accomplished (MacDonald, Spalding and Dholakia 2003). Optical tweezer is a direct optical particle manipulation which uses focused beam of photons to trap particles in certain position (Li et al. 2016).

Acoustophoresis is the application of ultrasonic sound waves to manipulate the particle positions. Different microfluidic devices can be used for cell separation using acoustophoresis e.g. silicon etched glass and glass capillary, acoustic separation and have a wide range of applications e.g. bead-based assay and bacteria separation (Ngamsom et al. 2016) (Yin et al. 2017).

1.10.2 Passive cell separation techniques

Passive cell separation is a technique that requires no external forces for cell separation. Here, mechanical (size) based filtration or interaction with the fluid flow is used, such as in deterministic lateral displacement (DLD) and inertial microfluidics.

Microfiltration technology is size-based passive separation that uses a variety of micro devices e.g. (pillar, wires), cross flow and hydrodynamic for particle filtration. Pillars and wires are mainly used for leukocyte separation from red blood cells (Zheng et al. 2007). The device consist of an inlet and main channel divided into sub-channels containing obstacles and outlets for leukocyte separation.

Inertial microfluidics based separation, where the cell separation only relies on the size of the cells, requiring no immuno-staining or antibody. The flow rate, sample concentration, and device geometry are usually the key factors for the successful separation using this method. However, the overlap in physical properties of the target and non-target cells interferes with the separation purity (Yang, Leong and Sohn 2015, Ryu et al. 2017).

Deterministic lateral displacement (DLD) is a technique, where the sample flows through an array of microposts where the array is arranged such that each row is slightly shifted. Particles with a size below a critical hydrodynamic diameter follow the periodic streamline patterns through the gaps, and are able to cross the posts Particles with a size above the critical hydrodynamic diameter cannot follow a streamline but bump against the posts, and are displaced laterally, opposite to the small particles (Huang et al. 2004).. Particles with different diameters can be separated and collected at different outlets. DLD has been used for

circulating tumor cell (CTC) separation (Liu et al. 2013). The DLD approach can effectively separate cells with different sizes, as well as cells with different shapes and deformability (Loutherback et al. 2012, Huang et al. 2004).

1.10.3 Affinity-based approach

Affinity-based cell separation approach is the most commonly used method to isolate target cells from a heterogeneous cell population, such as whole blood. Immunoaffinity is a classic cell isolation approach that uses an antibody as the affinity ligand to specifically retain one cell type with higher purity, while other cells are passed through the device. In immunoaffinity cell isolation, the microfluidic device is modified with an antibody specific to the target cells for isolation. Different subtypes of leukocytes have a unique combination of surface markers like CD3, CD4, CD8, CD18 etc, and can be targeted to specifically isolate cell subpopulations from whole blood. Microfluidic immunoaffinity based cell enrichment has been successfully performed to isolate (CD4+T cells) from HIV patients' whole blood (Cheng et al. 2009, Wang et al. 2011). This technique has also been used for the isolation of rare cells from whole blood such as CTCs for the early detection of metastatic malignancies (Nagrath, et al. 2007, Deng, et al. 2014; Pratt, et al. 2011). The strong interaction between cells and a surface-bound affinity molecule is required for optimal capture efficiency. Therefore, the orientation of the immobilized antibody is an important factor for efficient antigen-antibody interaction for cell capture (Dong et al. 2013). Another factor that should be considered in microfluidic immunoaffinity based capture approach is the change in the cell-antibody adhesion mechanics with the shear stress in the flow. Different cell types have different optimal shear stress standards for efficient capture. A Hele-Shaw device is normally used for analyzing and mapping of shear stress, where the unique geometry enables the shear stress to be decreased linearly over the length of the device (Usami et al. 1993) (Figure 5). The shear stress depends on the geometry of microfluidic channels and calculated by the formula below, where the flow rate of the fluid is optimized to maintain the target cell adhesion. Microfluidic chip design has been developed over time to improve cell capture efficiency, based on channel geometry and shear stress in micro-devices e.g. cancer cell capture devices (Stott et al. 2010, Karabacak et al. 2014).

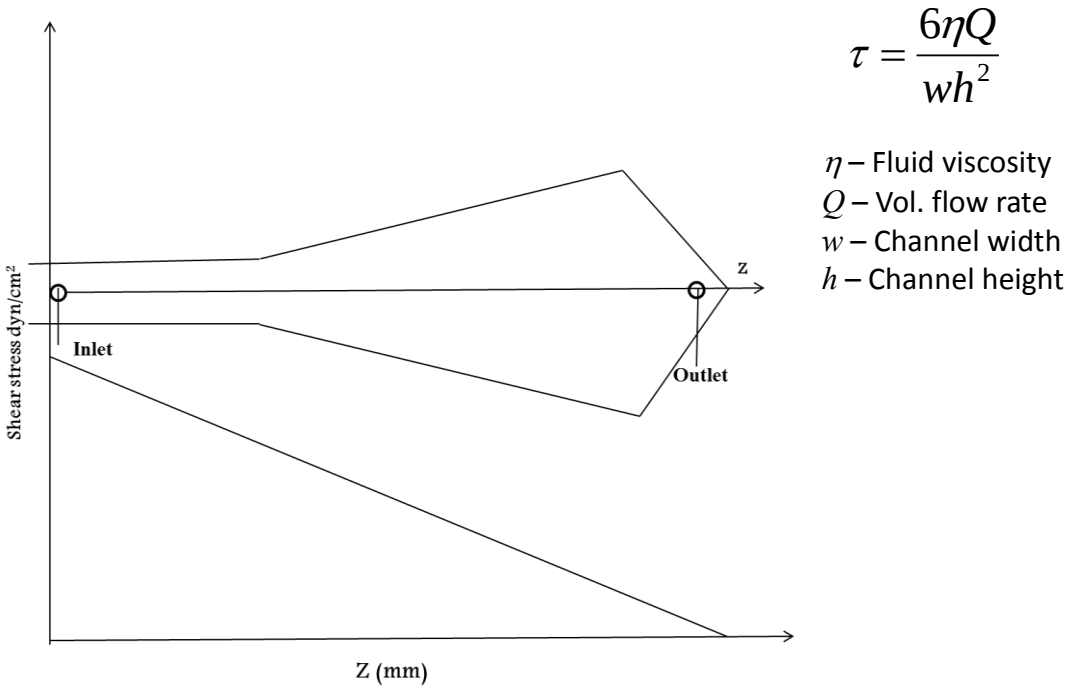


Figure 5*. Schematic illustration of a Hele-Shaw flow device. The shear stress decreases linearly over the length of the device as a consequence of the increased cross-section area.*

1.11 MICROFUIDIC-BASED LAB-ON-CHIP FOR CELL BIOLOGICAL ANALYSIS

Microfluidic technology has been used to study a variety of immune cell functions. Microfluidics has mostly been employed to investigate the cell migration and chemotaxis function of a broader range of cell types such as neutrophil and dendritic cells (DC). Some microfluidic devices have been developed for clinical applications. Neutrophils chemotaxis has been studied using chemoattractants such as (fMLP) and interleukin-8 (IL-8) where the cell morphology and migration responses were observed overtime in a microfluidic chip device (Wu et al. 2015). DC migration and trafficking in secondary lymphoid tissues is regulated by several chemokines, including CCL19 and CCL21, which have been employed in a flow-based microfluidic device to examine DC chemotaxis (Ricart et al. 2011). Moreover, Microfluidic technology has also been used to isolate neutrophil from whole blood for proteomics and genomic analysis (Kotz et al. 2010).

1.11.1 Microfluidic based basophil functional analysis

Recently, several microfluidic chip devices have been developed to study basophil function mainly related to allergic inflammatory responses, using basophil cell lines and bead-based basophil isolated cells. The basophil FcεRI activation pathway has been studied in an integrated microfluidic chip device. The Syk protein phosphorylation, calcium mobilization and the release of inflammatory mediators were monitored using cultured RBL-2H3 cells (Liu et al. 2013). Degranulation of allergen-treated basophil cell line KU812 cells has been studied using centrifugal microfluidics, using acridine orange (AO) fluorescent to stains the cell granules. Upon activation of cells by the stimuli, AO is released from the granules into the extracellular buffer through degranulation in a Ca2+-dependent manner. The drug screening to suppress the fMLP-mediated degranulation in basophils was recently

examined using an integrated centrifugal microfluidic platform (Kwok et al. 2016, Chen et al. 2012). Furthermore, a chip-cytometry technique has been used for quantification of bead-based purified basophils from bronchoalveolar lavage (BAL) and blood in asthmatic patients(Dijkstra et al. 2014).

2 AIMS OF THE THESIS

The general aim of the thesis is to investigate the role of the basophil in immune regulation in two inflammation driven diseases, chronic kidney disease and allergy. This is done by a newly developed microfluidic- based lab-on-chip technology together with the conventional methods.

The respective aims of the four studies are:

Paper I. To study the consequence of different hemo-dialyzer membranes on basophil activation in chronic kidney disease (CKD) patients.

Paper II. To study the basophil responsiveness towards different microbial antigens in CKD patients assessed by adhesion molecule expression and degranulation.

Paper III. To develop a novel method, microfluidic immune-affinity Basophil Activation Test (miBAT), to study the regulation of degranulation markers on basophils in allergic patients.

Paper IV. To assess the miBAT platform to be used as a reliable diagnostic method for allergy.

3 MATERIAL AND METHODS

This chapter describes the study population (patients and healthy controls) and the methods used in the studies of the thesis. More detailed descriptions can be found in the material and methods of the respective four papers (I-IV).

3.1 STUDY POPULATION

3.1.1 Patient characteristics

Study subjects from two different populations are included in this thesis. Patients in studies I and II were recruited from the Department of Nephrology at Karolinska University Hospital Solna, Stockholm, Sweden, and patients in studies III and IV were recruited from the Sachs's Children and Youth Hospital, Stockholm, Sweden. Written informed consent was obtained from all participants and the local Ethics Committee in Stockholm, Sweden, approved all the studies.

3.1.1.1 Study population in paper I

The patients (n=10) had an estimated Glomerular Filtration Rate (eGFR) of <20 ml/min/1.73 m^2 (stage 5D) with a residual GFR from 6-11 (median 7). Patients were undergoing hemodialysis with polysulfone high flux dialyzers, effective surface area of 2.3 m^2, K0A urea: 1421 mL/min, ultrafiltration coefficient: 76 mL/h/mm Hg, three times per week for four to four and a half hours per dialysis before the study. The dialyzer membrane was shifted from a high-flux to a low-flux polysulfone capillary dialyzer (effective surface area of 1.8 m^2, K0A urea: 976 mL/min, ultrafiltration coefficient: 14 mL/h/mm Hg). An arteriovenous fistula or a central dialysis catheter was used for dialysis.
Patients with cancer, an ongoing infection, chronic inflammatory disease and those taking immunosuppressive drugs were excluded.

3.1.1.2 Study population in paper II

Patients (n=10) with CKD stage 5D. The residual GFR ranged from 4-12 mL/min/1.73 m^2 (median 8.5). Patients were undergoing maintenance hemodialysis for four hours/session, three times per week, using polysulfone high-flux.

Patients with cancer, an ongoing infection, chronic inflammatory disease and those taking immunosuppressive drugs were excluded.

3.1.1.3 Study population in papers III and IV

In paper III eight allergic patients were included in the study; they were allergic to different allergens. Patients were clinically diagnosed with asthma, rhinitis and conjunctivitis. In paper IV patients (n=7) allergic to at least one of the airborne allergens were recruited. Patients on oral steroids and Allergen-Specific Immuno Therapy (ASIT) were excluded.

3.1.2 Healthy controls

In studies I and II the healthy controls (n=10) were recruited among healthy blood donors and were age and sex-matched (±5-7 years) with the patients. In studies III and IV healthy blood donors (n=7) were recruited from the Blood Center, Stockholm, Sweden.

3.2 BASOPHIL CELL LINE (KU812) CULTURE

Human basophil cell line (KU812) was used in paper III. Cells were cultured in RPMI1640 media containing 10% FBS (Fetal bovine serum) and 0.2% non-essential amino acids (Sigma Aldrich, Germany). Cells were cultured in a CO_2 incubator and the medium was renewed every two to three days through standard cell culture practice.

3.3 METHODS OF BASOPHIL ANALYSIS

3.3.1 Flow cytometric analysis of basophil

3.3.1.1 Paper I

Peripheral blood samples were collected in heparin tubes before and after dialysis process in each session (one occasion with a high-flux and one occasion with a low-flux dialyzer).

3.3.1.1.1 Basophil activation analysis

Basophil expression of degranulation markers (CD63 and CD203c) (Beckman Coulter) was analyzed by flow cytometry (Navios, Beckman Coulter), after activation of whole blood from patients and healthy controls with formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Aldrich), and anti-Fcε receptor I (FcεRI) antibody (Bühlmann Laboratories).

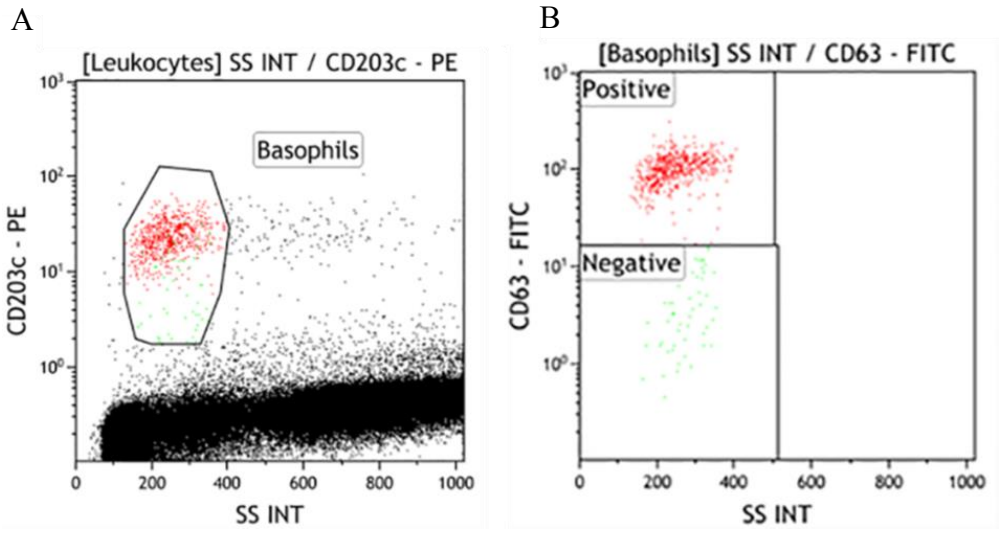


Figure 6. *Basophil gating strategy* ***(A)*** *Basophils were gated according to their granularity on side scatter and expression of CD203c.* ***(B)*** *CD63 positive cells were detected within the total basophil population.*

3.3.1.1.2 Basophil counting

The absolute number of basophils was analyzed before and after dialysis sessions using the ImmunoPrep reagent system (Beckman Coulter), cell-counting beads (Flow-Count Fluorospheres, Beckman Coulter), and analyzed by flow cytometry (Navios).

3.3.1.1.3 Adhesive markers analysis on neutrophils

Surface expression of CD11b (Beckman Coulter) and active CD11b (Biolegand) on neutrophils was analyzed by flow cytometry (Navios), following treatment of whole blood with IL-8 (CXCL8) (Research & Diagnostics Systems).

3.3.1.1.4 Expression of CD88 (C5aR) on neutrophils

Whole blood was incubated with anti-CD88 antibody (Becton Dickinson), and expression of CD88 was detected by flow cytometry (Navios).

3.3.1.2 Paper II

Peripheral blood samples were drawn into heparin tubes from hemodialysis patients before the start of hemodialysis sessions.

3.3.1.2.1 Adhesive markers expression on basophils

Basophil expression of CD11b, active CD11b and CD62L (Becton Dickinson) was detected by flow cytometry (Navios), following activation of different basophil activation pathways using Lipopolysaccharide (LPS) (Sigma Aldrich, Germany), (fMLP), Peptidoglycan (PGN) (Sigma Aldrich, Germany) and anti-(FcεRI) antibody in blood from patients and healthy controls.

3.3.1.2.2 Degranulation markers analysis on basophils

Flow cytometric analysis of CD203c, CD63 and CD300a (Bio Rad) expression on basophil cell surface was done after activation of different basophil activation pathways (FcεRI, TLR and formyl-peptide receptor pathways).

3.3.1.2.3 Expression of CD61 (platelet aggregation marker) on basophils

The experiment was done to rule out the possibility that the change of CD63 expression was due to platelet aggregation and not basophil activation. Healthy whole blood was treated with anti-FcεRI antibody; RPMI 1640 was used as negative control. CD63 and CD61 (MACS Miltenyi Biotech) expression on basophils was analyzed by flow cytometry (Navios).

3.3.1.2.4 CD62L blocking on basophil surface

The specificity of anti-CD62L binding in basophils was examined by the blocking of CD62L on the surface of basophils. Blood from healthy donor was incubated with (fMLP) and RPMI 1640 as a negative control. CD62L was blocked on basophil surface with un-

conjugated anti-CD62L (Abcam) and cells subsequently stained with anti-CD62L PE-cy5 (Becton Dickinson) and analyzed by flow cytometry (Navios).

3.3.1.3 Papers III and IV

3.3.1.3.1 Flow cytometric Basophil activation test

In paper III, flow cytometry analysis of CD63 and CD203c (Beckman Coulter) expression was performed, after activation of whole blood from allergic and healthy individuals with anti-FcεRI (Bühlmann Laboratories). RPMI (Sigma Aldrich) was used as negative control.

3.3.1.3.2 Flow cytometric analysis of basophil allergen threshold sensitivity

In paper IV, the flow cytometry analysis of CD63 expression was performed after basophil activation with the relevant airborne allergen and non-relevant allergens concentrations 1:10 dilution (5000, 500, 50 and 5 SQU/ml) (Aquagen, ALK, Copenhagen, Denmark). Anti-FcεRI was used as positive control and stimulation buffer as negative control.

3.3.2 Immunocytochemistry of CD62L on basophils and neutrophils

Immunocytochemistry technique was used in paper II. Whole blood was collected from a healthy donor and basophils were purified by MACS Basophil isolation kit (MACS, Miltenyi Biotec, Germany). Basophils were activated with fMLP and RPMI was used as negative control. Cells were fixed with paraformaldehyde (PFA) followed by incubation of the cells in blocking solution 1% bovine serum albumin (BSA). Cells were stained with primary unconjugated CD203c and CD62L then stained with secondary antibodies, stained basophils visualized by Eclipse Ti Nikon microscope. Images were transferred using MicroManager Version 1.4 software, plugged-in and processed using Imagej software. Neutrophils were activated with (fMLP) and stained with primary CD66b (MACS biotech) and anti-CD62L for microscopy imaging.

3.3.3 Serological analysis of serum IgE antibodies

In paper IV, sIgE level was analyzed in patients and healthy controls. Serum samples were sent to the diagnostic laboratory at the Karolinska University Laboratory. IgE-antibodies (IgE-ab) to allergens (timothy, birch, cat, dog, horse, mite and bee) were analysed using ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's instructions.

3.3.4 Microfluidic approach to basophil analysis

In papers III and IV microfluidic chip technology was used for functional characterization of basophils regarding the expression of CD63 and CD203c and detected by fluorescent microscopy. Blood was collected from allergic patients and healthy controls.

3.3.4.1 Chip microfabrication

The microfluidic device was used in the experimental setup of papers III and IV and comprises a straight channel, where the width, height and length of the channel were 4 mm, 50 µm and 25 mm respectively. A microfluidic device was fabricated in polydimethylsiloxane (PDMS) using standard soft lithography techniques (Xia et al. 1999). Briefly, channel replicas were produced on a silicon master by spin-coating, followed by UV exposure and developing to define the channel structures on the master substrate. The elastomeric PDMS (Dow Corning) was mixed with a cross-linker with a ratio of 10:1(wt/wt), and poured onto the master and cured at 65°C. The curved PDMS with replicated channels was peeled off from the silicon wafer and the inlet and outlet holes were punched before the channels were bonded to a glass slide.

3.3.4.2 Microfluidic chip surface modification

The channels in the chip surface were modified, initially by 3-mercaptopropyl trimethoxysilane chemistry (Sigma Aldrich, Germany), followed by washing with ethanol and the addition of 4-Maleimidobutyric acid N-hydroxysuccinimide ester (GMBS) (Sigma Aldrich, Germany), a cross linking agent, then Neutravidin (Sigma Aldrich, Germany) was infused into the chips. Finally, biotinylated CD203c (MACS, Miltenyi Biotech, Germany), the antibody specific for basophils, was immobilized on the surface of the chips.

3.3.5 Basophil capture characterization in microfluidic chip

3.3.5.1 Basophil capture from basophil cell line (KU812)

Straight channel capturing device was used to characterize basophil capture using basophil cell line (KU812) in paper III. The cells were washed and resuspended in 1xPBS for processing into the chip. 1% BSA (bovine serum albumin) (w/v) was pumped into the device surface at 20 µl min^{-1}. 70 µl of the sample was pumped into the straight channel chip at chosen flow rates (1-20) µl min^{-1} using a syringe pump (Harvard apparatus, USA). The unbound cells were washed-out with 1% BSA at 20 µl min^{-1}. Captured cells were stained using nuclear staining (Hoechst stain) (Sigma Aldrich, Germany), fluorescent images were taken by fluorescent microscope at different points all over the chips.

3.3.5.2 Basophil capture from whole blood

In paper III whole blood from healthy donors was processed into the capturing device at different flow rates (3-10) µl min^{-1}, and the device was washed with 1% BSA at a flow rate of 20 µl min^{-1}. The captured cells were stained using nuclear staining (Hoechst stain). In addition, CD203c was used to stain basophils in chips; fluorescent imaging of chips was done by fluorescent microscopy. Depletion assays were done by flow cytometry, basophil (CD203c) cells were counted in the aliquots collected before and after the passage of blood through the microfluidic device. The optimized flow rate was used for the subsequent experiments in papers III and IV (Figure 7).

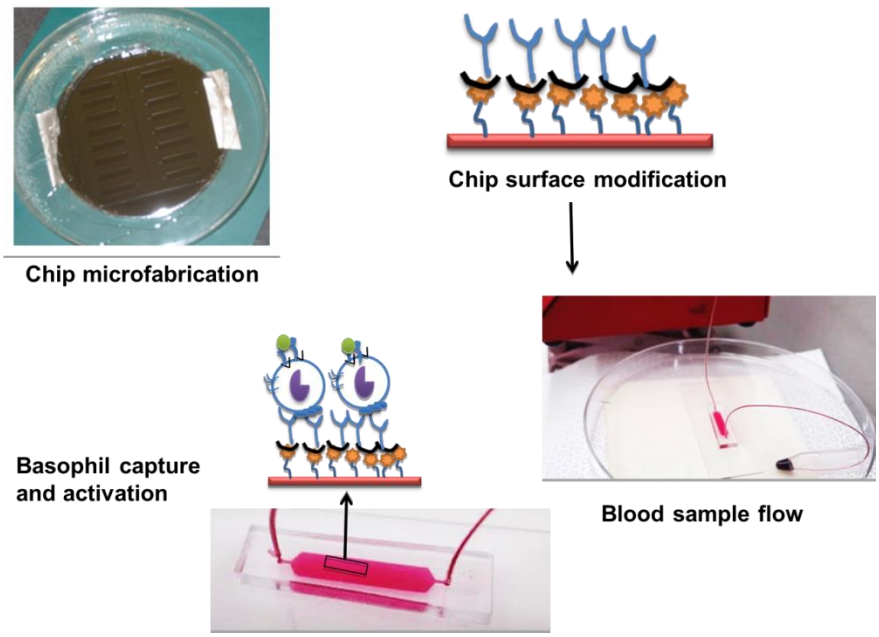


Figure 7. *Immunoaffinity basophil capture and activation of basophils in microfluidic chip.*

3.3.6 Basophil activation analysis in microfluidic chip

3.3.6.1 Basophil activation in microfluidic chip with anti-FcεRI antibody

In paper III, basophils captured in the chip were activated with anti-FcεRI antibody (Bühlmann Laboratories) at 37^{o}C. 1% BSA was pumped into the negative control chip. The captured cells in the chips were stained with CD63 and CD203c and expression of these markers was analyzed by fluorescent microscopy. The experiments were performed on blood from healthy donors and allergic patients.

3.3.6.2 Basophil activation in microfluidic chip with airborne allergens

In paper IV, captured basophils in chips were challenged with two concentrations (5000-50 SQU/ml) of relevant airborne allergens (presence of IgE-ab) (birch, timothy, cat, dog or horse). A non-relevant allergen (absence of IgE-ab) (dust mite and bee) was used as control allergen. In addition a negative control (stimulation buffer) and a positive control (anti-FcεRI antibody) were analyzed. CD203c and CD63 expression on captured basophils was thereafter detected by fluorescent microscopy. The experiments were performed on blood from healthy donors and allergic patients.

3.4 STATISCAL ANALYSIS

Statistical analysis in studies I-IV was done in GraphPad Prism 5 (GraphPad Software), Wilcoxon matched-pairs signed rank test for two dependent measurements (paper I), and Mann-Whitney U test for two independent sample groups (papers II, III, IV) was applied to analyze non-normally distributed values. For comparison of three independent measurements nonparametric Kruskal-Wallis test was carried out (papers I, II).

4 RESULTS AND DISCUSSION

4.1 BASOPHIL ACTIVATION AND BIOCOMPATIBILITY EVALUATION IN HEMODIALYSIS (PAPER I)

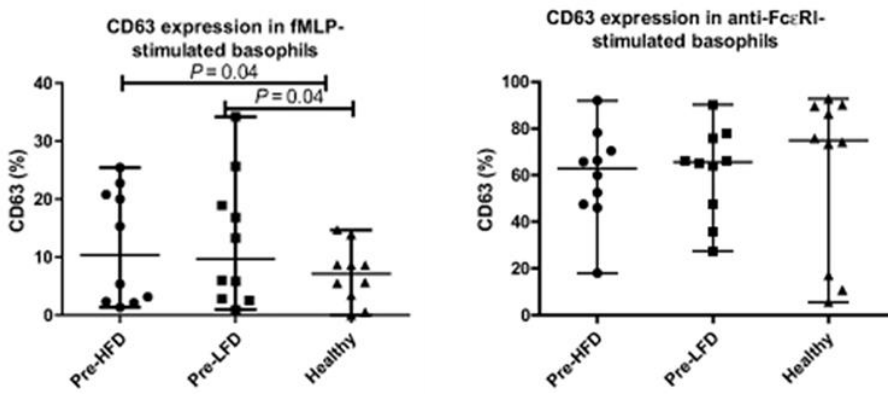
Hemodialysis is a process in which the accumulated uremic toxins, minerals and excessive fluid in end stage renal disease (ESRD) are removed from the blood by diffusion and filtration of the blood within the dialyzer. The interaction between blood and dialyzer membranes leads to alteration in immune cell function and circulating plasma proteins (Banche et al. 2006, Chauveau et al. 2005). The bioincompatibility of dialyzer membranes is dependent on the surface material and the membrane permeability (Craddock et al. 1977). In this paper, we hypothesized that the passage of basophils and neutrophils through the dialyzer affects basophil and neutrophil activation and that the effect on passing cells may differ between different pore sized dialyzers (high flux and low flux).

Basophils were stimulated with fMLP and anti-FcεRI antibody, and basophil CD63 expression was analyzed using flow cytometry. Venous blood samples were collected from CKD-patients before and after each session of dialysis using both dialyzers, and compared to blood collected from healthy controls. We observed that CD63 expression in fMLP-activated basophils in hemodialysis patients was significantly higher compared to healthy controls ($p= 0.04$) (Figure 8A). However, this was not the case following stimulation with anti-FcεRI antibody.

It is not clear whether the underlying mechanism of increased fMLP responsiveness is a consequence of altered fMLP receptor expression (formyl peptide receptor (FPR) or formyl peptide receptor-like 1 (FPRL1)), or whether the intracellular pathways (MEK–ERK pathway), involved in degranulation, are primed in basophils from CKD patients (Miura and MacGlashan 2000, de Paulis et al. 2004). Therefore, further analysis for better understanding of the underlying mechanisms involved in the increased fMLP responsiveness is needed.

The CD63 expression on fMLP-activated basophils was significantly higher ($p = 0.01$) after dialysis with low-flux dialyzers compared with high-flux dialyzers. Anti-FcεRI antibody-activated basophils' CD63 expression was significantly increased ($p = 0.002$) after dialysis with both dialyzers (Figure 8B). In comparison between pre- and postdialysis in both dialyzers, CD63 expression was significantly higher on anti-FcεRI antibody-activated basophils following hemodialysis with low-flux membranes ($P = 0.002$) (Figure 8B).

A



B

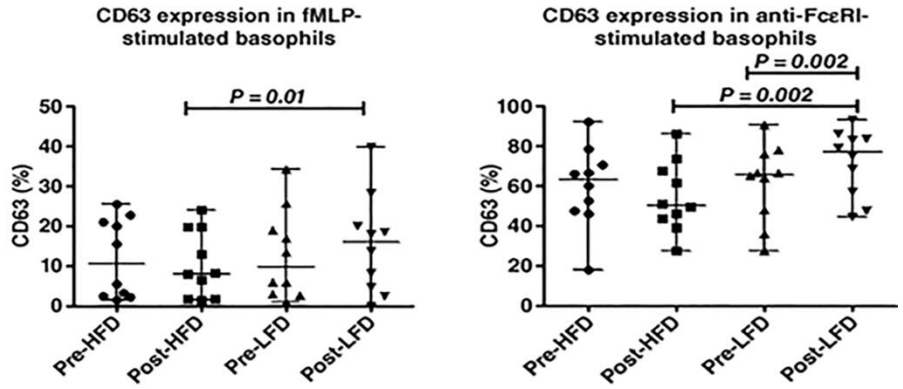


Figure 8. *Basophil activation (CD63 expression). (A) CD63 expression on basophils after fMLP and anti-FcεRI antibody comparing patients (pre dialysis) with healthy controls. (B) CD63 expression in activated basophils following dialysis with low-flux and high-flux dialyzers.*

The differences in FcεRI-mediated activation of basophils were detected following basophil passage through low flux polysulfone dialyzer but not through highflux dialyzer. Patients undergoing hemodialysis may develop anaphylactic reactions. The underlying mechanism involved in hemodialysis-associated anaphylactic reactions can be that the cross linking of FcεRI on basophils results in a stronger response and release of immune-mediators such as histamine and heparin (Ebo et al. 2006). It has also been shown that different membrane surface charges can lead to activation of the Hageman factor and bradykinin which may activate basophils in an IgE-independent manner and induce histamine release followed by anaphylactic reactions during hemodialysis (Verresen et al. 1994). In this study, we suggest that the different pore size and ultrafiltration coefficient of the dialyzer membrane may have an impact on the priming of the basophils and their activation. This supports the notion that high-flux dialyzers may be favorable to have an advantage to low-flux dialyzers in this respect (Locatelli 2003, Eknoyan et al. 2002). Immune-mediators secreted from other cells during hemodialysis can also activate basophils, e.g. IL-1β has been shown to induce histamine release from basophils (Subramanian and Bray 1987).

Furthermore, the absolute number of basophils, before and after dialysis with either dialyzer, was unchanged compared to healthy controls. This indicates that the alteration of basophil

activation was due to functional variations and not to the entrapment of basophils in the dialyzer.

CD11b and active CD11b (MAC-1) expression on neutrophils were analyzed. Active CD11b (MAC-1) is involved in neutrophil adhesion to endothelial cells, and transmigration toward the inflammation site. It has been shown that neutrophil CD11b expression increases during the hemodialysis procedure, using either low- or high-flux membranes. The transmigration process of neutrophils changes after dialysis by low-flux membranes (Moshfegh et al. 2002). In this study, we analyzed the surface expression of the CD11b active epitope on neutrophils. There were no significant differences in CD11b expression (MFI) on activated neutrophils when comparing dialysis patients with healthy controls and comparing both dialyzers.

Moreover, we analyzed the expression of C5aR (CD88) on neutrophils. Anaphylatoxin C5a has been shown to be induced to various degrees by hemodialysis (Erlenkotter et al. 2008). C5a binds to C5aR and the interaction leads to cleavage of the receptor from the surface of the cells (Van den Berg et al. 2014), which indirectly indicates cell exposure to the anaphylatoxin. No significant differences were observed of CD88 expression on neutrophils either before or after hemodialysis. This could be due to the difference in the number of study subjects or the insensitivity of this molecule to assessment of bioincompatibility.

In summary, in paper I we showed that basophil activation (CD63 expression) was significantly altered following low-flux hemodialysis and also in hemodialysis patients compared to healthy controls. In contrast, no difference was detected in CD11b and active epitope CD11b expression on neutrophils. This may suggest a role for the basophils as a new marker for evaluation of dialyzer membranes biocompatibility.

4.2 BASOPHIL DYSFUNCTION IN CHRONIC KIDNEY DISEASE PATIENTS (STAGE 5D) (PAPER II)

Patients with end stage renal disease (ESRD) on hemodialysis have a high risk of morbidity and mortality, which is mainly associated with cardiovascular disease and infections (Epstein 2015, Go et al. 2004). Basophils have been shown to have a potential role in immune function disturbances in CKD patients either due to retention of toxins or effect of hemodialysis (Vaziri et al. 2012, Bosch et al. 2011). In paper I we demonstrated that basophil responsiveness to bacterial peptide (fMLP) in hemodialysis patients is increased compared to healthy controls. Therefore, the aim of study II was to further investigate the potential basophil function alteration towards microbial antigen exposure, referred to adhesion molecule expression and degranulation in CKD patients on hemodialysis. For the transmigration process we selected the adhesion molecules CD11b, active epitope CD11b and CD62L and for the degranulation process CD203c (piecemeal degranulation marker), CD63 (degranulation marker), and CD300a (inhibitory marker of degranulation). We stimulated different basophil activation pathways, (TLR, Formyl-peptide receptor and FcεRI pathways).

Basophil expression of active CD11b (Mac-1) MFI was significantly increased (p=0.005) after LPS activation (TLR4) in patients compared to healthy controls. In contrast, there was no difference in the basophil expression of active CD11b epitope after TLR2 (PGN), fMLP or anti-FcεRI activation (Figure 9A).

Previous studies reported that basophils have a relatively high expression of TLR-4 mRNA compared with the other TLRs (Suurmond et al. 2014a, Komiya et al. 2006). This corroborates our obtained data, which found that basophil responsiveness was higher after TLR4 activation than TLR2. Interestingly, basophils expressed significantly higher levels of active epitope CD11b which is more relevant to CD11b functional capacity; it is known that active epitope CD11b was formed due to a conformational activation of total CD11b on the cell surface, and facilitated the actual binding (Oxvig, Lu and Springer 1999, Arnaout 1990). This finding suggests that the basophils more readily adhere to endothelium and extravascular matrix after exposure to LPS. LPS-activated basophils secret pro-inflammatory cytokines e.g. IL-1 and TNF-α, which may be involved in the inflammatory state in CKD patients (Suurmond et al. 2014a).

An interesting finding was that the CD62L expression in basophils was stable after activation with LPS, PGN, fMLP and anti-FcεRI-ab. This was in contrast to the down-regulation of CD62L MFI expression detected in activated neutrophils in hemodialysis patients. To examine the stability of CD62L, we performed a blocking experiment of CD62L in basophils (Figure 9B). Immunocytochemistry staining of CD62L in basophils and neutrophils before and after activation shows conserved expression of CD62L in basophils after stimulation compared with down-regulated CD62L in stimulated neutrophils. Moreover, CD62L was significantly down-regulated (p=0.04) after activation with anti FcεRI-ab in healthy controls, while, there was no significant difference in CD62L expression in comparison between patients and healthy controls.

The CD62L expression in basophils is stable, in contrast to neutrophils and monocytes where it is rapidly shed from cell surface after activation (Monteseirin et al. 2005, Xu et al. 2008). The cytoplasmic tail of CD62L interacts with at least three different proteins and disruption of these interactions may reduce the shedding, which may prohibit the rolling capacity in basophil (Jung and Dailey 1990, Dwir et al. 2001). We observed down-regulation of CD62L in healthy controls after activation with anti-FcεRI. A rationale for this observation is that stimulation with anti-FcεRI enhances production of immune-mediators such as TNF-α which modulates CD62L shedding (Thyagarajan et al. 2012).

CD63 and CD300a expression in fMLP-activated basophils was significantly higher in hemodialysis patients compared to healthy controls (p=0.04 and p=0.01, respectively). Moreover, the expression of CD63 after activation with anti-FcεRI antibody was not significantly different in patients compared to healthy controls, while the CD300a expression was significantly higher in patients (p=0.01) (Figure 9C). We observed that stimulation of TLRs in basophils did not upregulate CD63 expression in patients or in healthy controls. Furthermore, CD203c MFI and the ratio between CD63 and CD300a were not significantly different in patients. The expression of CD61 (platelet aggregation marker) was not significantly different in basophils after activation, indicating that the change of

CD63 regulation in basophils was due to basophil degranulation and not aggregation of platelet on the basophil surface.

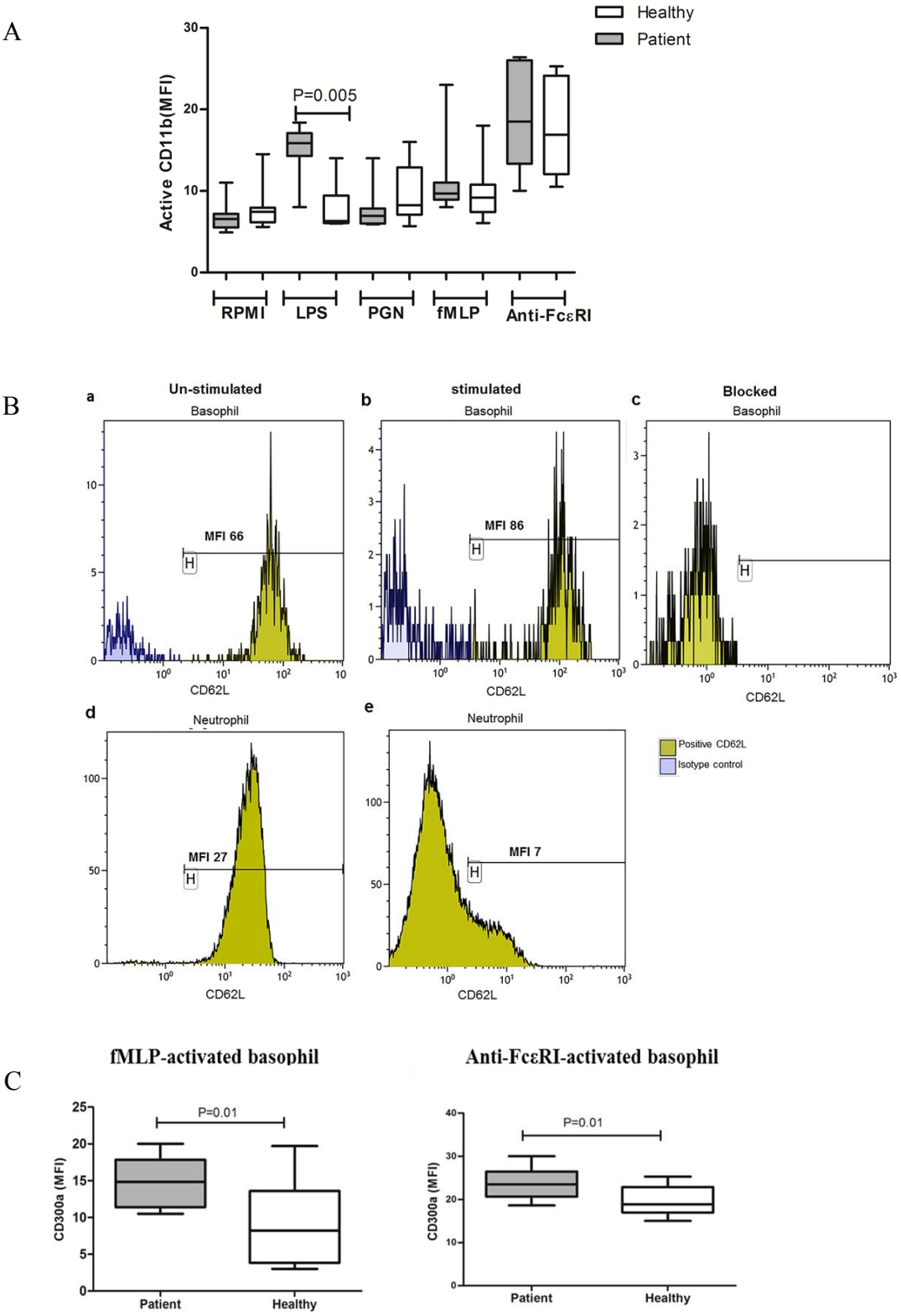


Figure 9. *Basophil expression of Active epitope CD11b, CD62L and CD300a (MFI). (A) Active epitope CD11b expression on basophils after stimulation of different pathways. (B) Flow cytometric analysis of CD62L on basophils and neutrophils. (C) CD300a expression in fMLP and anti-FcεRI-activated basophils.*

Activation of TLR did not upregulate CD63 in basophils. Previously, it has been shown that activation of basophil TLRs did not induce degranulation but did induce secretion of cytokines e.g. IL-4(Gyimesi et al. 2013). CD300a is an inhibitory marker for degranulation (Gibbs et al. 2006) and was significantly higher in patients as compared to healthy controls. The higher level of CD300a expression might indicate that the regulatory mechanism is in action as a response to increased CD63 expression.

In summary, in paper II we showed that activation of basophil pathways related to exposure of microbial molecules, and significantly increased expression of the CD11b active epitope and the stability of CD62L expression, and this indicates that basophils in CKD patients have an impact on adhesion and transmigration. Moreover, upregulation of degranulation markers on basophils proposes an altered basophil degranulation process. Altogether, these results indicate the potential role of basophil in CKD patients.

4.3 MICROFLUIDIC IMMUNOAFFINITY APPROACH - A NEW PLATFORM TO PERFORM BASOPHIL ACTIVATION TEST (PAPER III)

IgE dependent basophil activation starts when an allergen cross-link IgE-ab is bound to FcϵRI on the basophil surface (Stone et al. 2010). Activation of FcϵRI enhances a number of downstream signaling events in the basophil leading to upregulation of intracellular Ca+ signaling which in turn induces cell degranulation, release of immune mediators, and upregulation of activator markers (CD63 and CD203c) on the basophil surface (Knol and Gibbs 2014). Detection and quantification of basophil activation markers can be done by flow cytometry. The flow cytometry based Basophil Activation Test (BAT) is used as a diagnostic tool for allergy but the complexity of the flow cytometry operation has limited use in the clinic. Therefore, the aim of paper III was to develop a novel method (microfluidic based immunoaffinity approach, miBAT) to perform a basophil activation test.

The microfluidic chip was designed, modified and characterized to capture basophils directly from whole blood. The device was initially characterized using a basophil cell line (KU812), to optimize the capture and washing flow rates. The optimal shear stress (flow rate) using the straight channel (50 μm x 4 mm height and width) corresponded to 3 μL min^{-1}. When the shear stress was increased, the cell capture efficiency decreased. The decline in cells captured at a higher flow rate indicated less time for antibody-cell interaction. The cells can endure a washing rate of 20 μL min^{-1} and the maximum cell adhesion was at 10 mm along the length of the chip (Figure 10A). Moreover, the specificity of cell capture was confirmed using control chips (without anti-CD203c coating).

Following the capture of basophil cell line in chips we moved on to capture basophils from whole blood. The highest basophil capture yield was at a flow rate of 3 μL min^{-1} and decreased gradually with higher flow rates. The yield was analyzed by flow cytometric analysis of the basophil counts before and after the flow of blood through the chip (Figure 10A). The capture purity, calculated as the ratio between the (CD203+ cells/total leukocyte), was approx. 40%. In a flow cytometry based basophil activation test the sufficient number of basophils is 200 (Sanz et al. 2002) and our device captured more than

this number. One ought to consider the impact of monocytes present due to their expression of a low level of FcεRI and CD63. However, the activation of the FcεRI pathway in monocytes is different and requires a higher concentration of stimuli and also longer incubation times to cross link FcεRI compared to basophils (Agis et al. 1996). Furthermore, captured basophils were also stained specifically with CD203c to exclude CD63 signals other than those from CD203c+cells.

Further, the miBAT assay was evaluated to establish whether it was able to detect expression of activation markers on captured basophils. The captured basophils were stimulated by an anti-FcεRI antibody followed by staining and detection of CD203c and CD63 expression using fluorescence microscopy. We noticed that the CD203c mean fluorescence intensity (MFI) in captured activated basophils was significantly higher ($p=0.02$) than the non-activated basophils in healthy individuals and in allergic patients ($p=0.04$) (Figure 10B). The results were parallel to the flow cytometry analysis. CD203c can be considered to be both an identification and activation marker of piecemeal degranulation (PMD) for basophils. CD203c is expressed constitutively on basophils and upregulated upon activation.

Expression of CD63 on anti-FcεRI-activated captured basophils was compared to non-activated basophils in microfluidic chip. We found that CD63% in basophils from allergic patients (median and range) 48 (36-69) % was significantly higher ($p=0.0002$) compared to non-activated captured basophils (negative control) which was 22 (10-33) %. The same pattern was observed when basophils were analyzed by flow cytometry ($p=0.0009$).

In microfluidic chip analysis we observed a relatively high basal in vitro value (background) in the negative control. The high background could be due to the presence of pyrogens and endotoxins that contaminated the materials such as plastic tubes or syringes used in the technique (Sanz et al. 2002). Therefore, it is important to work in a sterile environment and redesign a chip with the possibility of minimizing the assay process time that might reduce the negative background. The mechanical stress formed during the cell capturing process in the chip may prime the spontaneous activation of captured basophils and induce degranulation (Paszkowiak and Dardik 2003, Boccafoschi et al. 2010). The background was further analyzed by measuring the CD63 MFI of anti-FcεRI-activated basophils compared to non-activated captured basophils. The CD63 MFI was significantly higher ($p=0.0001$) in activated basophils than in non-activated captured cells, suggesting that the expression level of CD63 in activated basophils on a single cell level is higher than in non-activated cells (Figure 10C). The CD63MFI ratio (CD63MFI of activated basophils/CD63MFI in non-activated basophils) has previously been used to quantify basophil activation and to set the basophil activation threshold (Hoffmann et al. 2015). Moreover, the CD63 expression was significantly higher in anti-FcεRI activated captured basophils ($p=0.03$) in allergic patients compared to healthy controls. This result was parallel to the flow cytometry analysis of CD63 expression comparing both groups ($p=0.04$). This might be due to the variation of the FcεRI expression level on basophils between allergic and non-allergic individuals, and may regulate the intensity of basophil degranulation and CD63 expression in basophils (Sihra et al. 1997). Furthermore, CD63 expression in activated captured basophils analyzed in microfluidic chips non-significantly differed

compared to flow cytometry analysis, indicating the sensitivity of miBAT technology to quantify basophil activation.

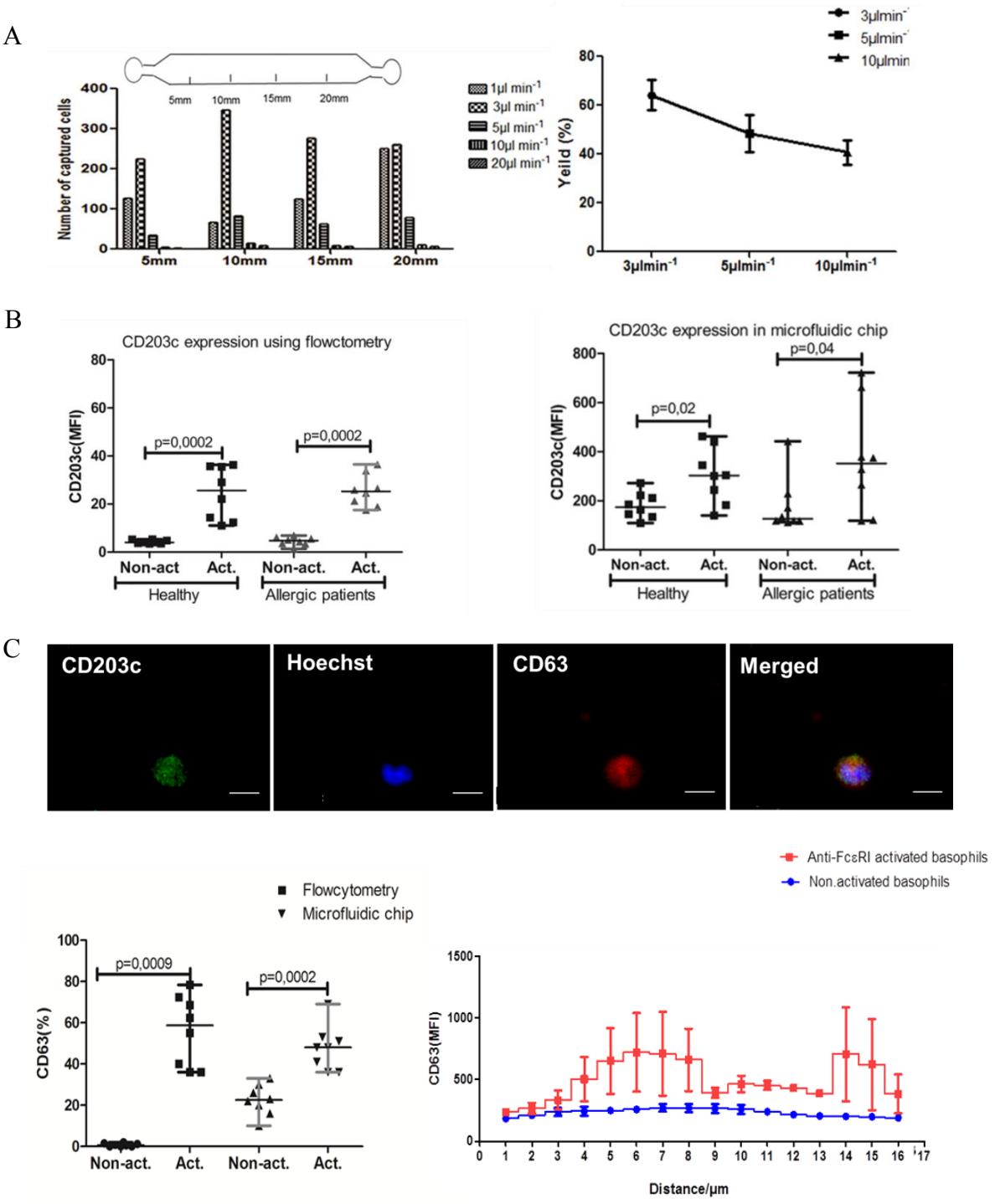


Figure 10. *Basophil capture and activation markers expression (CD203c, CD63) in microfluidic chip. (A) Microfluidic chip characterization using basophil cell line (KU812) and whole blood. (B) CD203c expression in anti-FcεRI-activated basophils compared to non-activated basophils in microfluidic chip and flow cytometry. (C) CD63 expression in anti-FcεRI-activated basophils compared to non-activated basophils in microfluidic chip and flow cytometry, fluorescent image of activated basophil in chip, histograms of CD63MFI in activated captured basophils and non-activated basophils.*

Altogether, the results presented in paper III show the ability of our miBAT technology to capture a sufficient number of basophils from whole blood, followed by detection of activation marker expression (CD203c, CD63) in anti-FcεRI-activated basophils. We also demonstrated that the data were comparable with flow cytometry analysis.

4.4 MICROFLUIDIC BASED BASOPHIL ACTIVATION TEST FOR ALLERGY DIAGNOSIS (PAPER IV)

Following the interesting results obtained by anti-FcεRI basophil stimulation in paper III, we further evaluated the microfluidic immunoaffinity BAT (miBAT) technique in paper IV for allergy diagnosis using allergens for activation.

Basophils captured in the miBAT device were challenged with two concentrations of airborne allergens, anti- FcεRI (positive control), and stimulation buffer (negative control) in allergic and non-allergic individuals.

We showed that CD63% expression in anti-FcεRI activated captured basophils was significantly higher ($p=0.003$) compared to CD63 expression in non-activated (negative control) basophils from allergic individuals. Moreover, CD63 expression on basophils from allergic patients was significantly higher after stimulation with the relevant allergen (5000 and 50 SQU/ml) as compared to the negative control ($p=0.01$, $p=0.003$, respectively). The CD63 expression in basophils activated with a non-relevant allergen (control allergen) gave results comparable to the negative control (Figure 11A). Results obtained from the healthy controls showed that the CD63 expression of anti-FcεRI activated basophils significantly differed ($p=0.005$) from the negative control. In addition, allergen-treated basophils from healthy controls were not significantly different in comparison with negative controls. Despite the high negative background it is still possible to detect basophil activation in microfluidic chip.

Flow cytometry analysis of CD63 expression in anti-FcεRI activated basophils from both allergic patients and healthy controls was significantly higher ($p=0.0006$, 0.004 respectively) compared to non-activated basophils. Furthermore, flow cytometry analysis of CD63 in allergen-activated basophils compared to negative controls in allergic patients was significantly higher ($p=0.006$).

Analysis of the CD63 mean fluorescence intensity (MFI) of allergen-activated basophils from allergic patients was significantly higher ($p=0.0001$) compared to non-activated captured basophils. This indicates that the level of CD63 expression in activated basophils on a single cell level is higher than in non-activated cells. This is in line with our previously obtained data regarding the CD63 MFI value in anti-FcεRI activated basophils. Therefore, the CD63MFI ratio (CD63MFI activated basophils/CD63MFI non-activated basophils) might be used to set the activation threshold to measure basophil activation.

Furthermore, allergen-activated basophils using different concentrations from allergic patients showed comparable CD63 expression in microfluidic chip and flow cytometry (Figure 11B). We observed that the CD63 expression in allergen (5000 and 50 SQU/ml) activated basophils was significantly higher ($p=0.003$) in allergic patients as compared to

healthy controls. This was in line with the flow cytometry analysis data (p=0.001) (Figure11C).

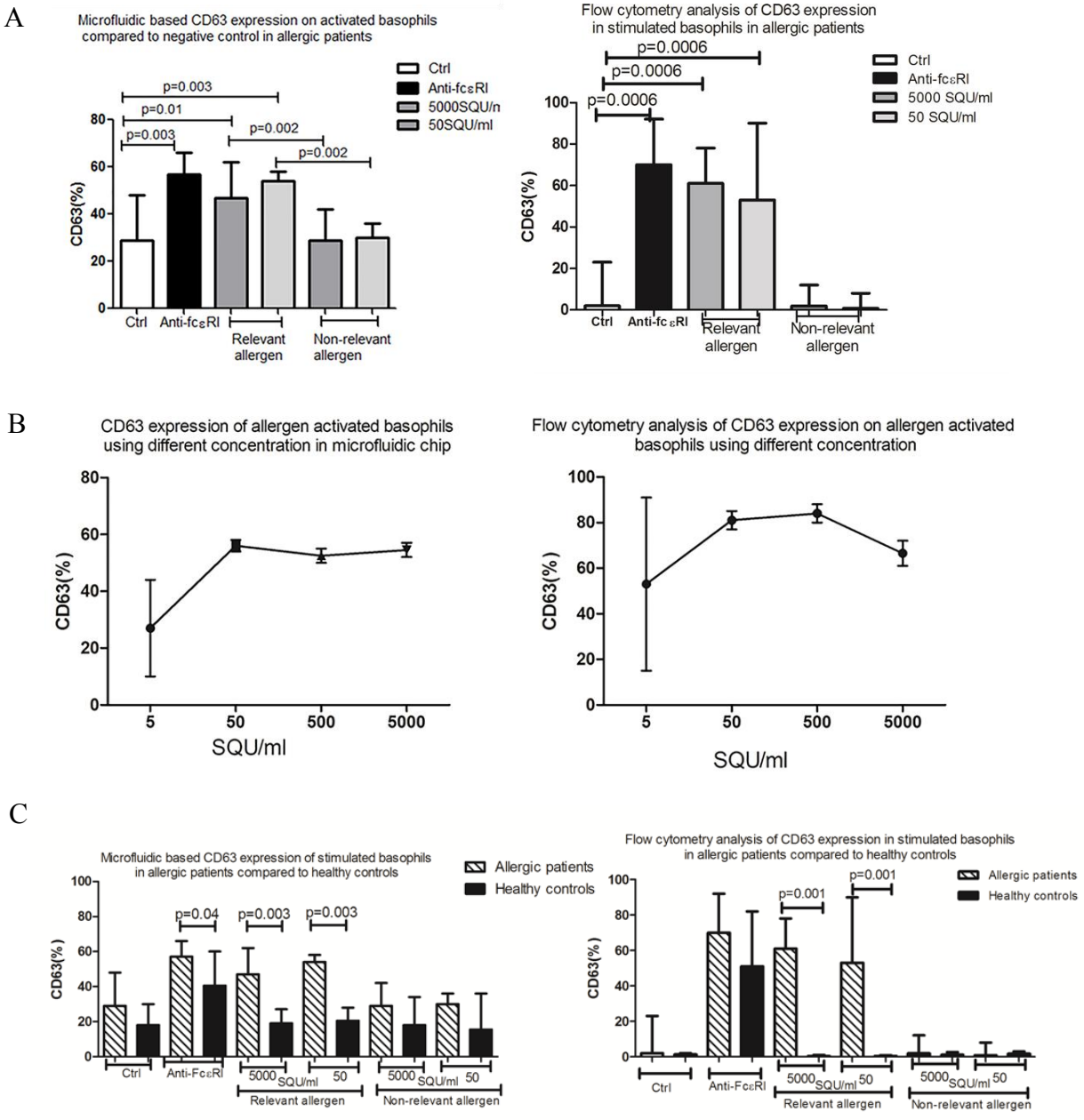


Figure 11. *CD63 expression in allergen activated basophils. (A) CD63 expression in anti-FcεRI and allergen-activated captured basophils compared to non-activated cells in allergic patients in microfluidic chip and flow cytometry. (B) CD63 expression in basophils after activation with different concentrations of allergen in microfluidic chip and flow cytometry. (C) CD63 expression in activated basophils from allergic and healthy individuals analyzed in microfluidic chip and flow cytometry.*

In summary, in paper IV we demonstrated that the miBAT technology was able to measure basophil activation after allergen exposure by quantifying their expression of CD63 in the microfluidic chip. The microfluidic chip discriminates between allergen activation and background as well as between stimulation with relevant and non-relevant allergens.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The vital role of the basophil in the immune regulation of inflammatory diseases has been given less attention compared to other immune cells. The research on basophils was hampered for a long time due to the rareness of basophils in peripheral blood which often caused difficulty in isolating them. However, during the last decade basophils have been given some recognition mainly in allergic diseases (Karasuyama et al. 2009). Basophils have also been shown to be involved in inflammatory responses of autoimmune kidney diseases, such as lupus nephritis (LN). Therefore, in the first part of this thesis, we addressed the question of whether basophil immune responses contributed to the inflammatory states of chronic kidney disease (ESRD) patients on hemodialysis, analyzed by conventional immunological methods.

Our studies have demonstrated for the first time that blood-dialyzer interaction impacts the basophils responsiveness in CKD patients on hemodialysis and that anti-FcεRI and fMLP stimulation is affected when the cells pass through low- or high-flux polysulfone dialyzers. An increased responsiveness to fMLP in patients on hemodialysis compared to healthy controls was also observed, Taken together, these data suggest that basophil activation may be a new marker for evaluating dialyzer biocompatibility properties.

In addition, we revealed that basophils in CKD patients (stage 5D) have an altered function judged by the regulation of transmigration and degranulation markers. A lack of CD62L shedding after basophil activation and upregulation of the CD11b active epitope in LPS-activated basophils was observed. This notion together with an upregulation of CD300a and CD63 expression in fMLP-activated basophils indicates altered basophil transmigration and degranulation processes in CKD patients. These data propose a potential role for the basophil in the pathogenesis of complications related to infection in chronic kidney disease patients on hemodialysis.

We reported that basophils contribute to inflammation and alteration mainly in innate immune responses in CKD patients on hemodialysis. However, the patho-physiological consequences of these observations warrant further studies. This is of importance for a deeper understanding of the underlying mechanisms of basophil functional alteration and the role of basophils in the state of immune deficiency in CKD patients that leads to severe complications e.g. recurrent infections, which can lead to further deterioration of the patient's condition and disease progression.

The techniques that are currently used for the purification of primary human basophils from whole blood or buffy coats are time-consuming and complex methods, which have greatly constrained in vitro studies of basophil biology. Therefore, in the second part of the thesis, we aimed to develop a novel microfluidic based method to isolate basophils directly from whole blood in a single step. The cells were thereafter subjected to analysis of the regulation of a degranulation marker in activated basophils in allergic patients and, in parallel, compared to flow cytometry analysis.

We developed a microfluidic based basophil activation test (miBAT), capable of isolating basophils from whole blood and detecting the activation markers' expression (CD203c and CD63) in anti-FcεRI activated captured basophils. We compared samples from both allergic patients and healthy controls. The miBAT results were comparable to flow cytometry data, which indicates the efficacy of our system to measure the basophil activation level by quantification of CD203c and CD63 expression in anti-FcεRI activated captured basophils. The flow cytometry-based basophil activation test is a method used in allergy diagnosis and monitoring. However, the complexity of the flow cytometry technique has limited its use in the clinic and we therefore presented the miBAT technology as a potential method to be used as a point of care for allergy diagnosis. Our study demonstrated that miBAT technology is able to detect the difference in degranulation marker expression (CD63%) in allergen-activated captured basophils compared to non-activated basophils in allergic patients. Moreover, increased CD63 expression in allergen-activated basophils in allergic patients compared to healthy controls was detected using miBAT. Overall, the results obtained using microfluidic chip analyses were comparable to flow cytometry analysis. Altogether, these data verified the efficacy of our device to be used to measure the level of basophils activation by quantifying their expression of CD63 in the microfluidic chip in allergic patients. The simplicity of this technique provides a new and consistent method to perform BAT using a microfluidic chip, which enables diagnosis and monitoring of allergic patients.

MiBAT is an interesting technology and, with further technical development, which shows great potential to be used for the clinical diagnosis of allergy or the monitoring of patients under treatment. Furthermore, the microfluidic chip represents valuable technology that can be employed in several applications either clinically or when studying biological cell functions

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